Ghent University

Faculty of Pharmaceutical Sciences

TREATMENT OF PERITONEAL CARCINOMATOSIS VIA (HYPERTHERMIC) INTRAPERITONEAL CHEMOTHERAPY: TOWARDS A UNIFIED STANDARD OF CARE

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Thesis submitted to obtain the degree of doctor in Pharmaceutical Sciences

2013

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Ghent, October 9th, 2013

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DANKWOORD

Aan een doctoraat werk je nooit alleen, hoorde ik bij het begin van mijn doctoraat. Tijdens de voorbije 5 jaar leek niks minder waar te zijn. Op de weg naar het neerleggen van dit werk, kwam ik heel wat mensen tegen die mij op de één of andere manier geholpen hebben. In het onderstaande stukje wil ik een aantal mensen in het bijzonder bedanken. Ook diegene die hieronder niet vermeld staan maar die toch op een enkele manier een bijdrage aan dit werk geleverd hebben wil ik langs deze weg bedanken!

Vooreerst wil ik mijn promotoren, Prof. Jean Paul Remon en Prof. Chris Vervaet bedanken voor de kans die ze mij gaven om te kunnen doctoreren. Maar ook voor hun begeleiding en het enthousiasme waarmee ze mij hebben begeleid tijdens dit voor hen niet steeds evidente project. Ook wil ik hen bedanken voor de mogelijkheid die ik gekregen heb om mee te werken aan verschillende andere Farm Tech projecten waardoor ik extra kennis heb kunnen opdoen. Ten laatste wil ik Chris in het bijzonder bedanken voor het kritisch nalezen van alle teksten, posters, artikels,…

Mijn promotor Prof. Wim Ceelen wil ik ook graag bedanken voor zijn intresse, de koppeling tussen het farmaceutische en het klinische, de begeleiding in de zaken waar je als apotheker weinig over weet en de vele interessante discussies tijdens mijn doctoraat.

Daar mijn project verschillende vakgebieden heeft overschreden heb ik ook met vele verschillende labo's samengewerkt. Graag wil ik alle labo's waar ik welkom ben geweest voor het uitvoeren van mijn experimenten bedanken. Het labo voor experimenteel kanker onderzoek in de P7 waar ik steeds al mijn cel-experimenten heb kunnen uitvoeren. De mensen van het Infinity Lab voor het helpen tijdens de MRI opnames en het ter beschikking stellen van hun software om de analyses uit te voeren. De mensen van het animalarium wil ik bedanken voor de goede zorgen die mijn ratjes van jullie hebben gekregen!

Ook wil ik Pieter Colin bedanken, dankzij onze samenwerking heb ik mijn resultaten op een andere manier leren bekijken! Nog veel succes met het afwerken van het farmacokinetisch model alsook met het afwerken van je doctoraat.

Al mijn dierexperimenten heb ik uitgevoerd in de kelder van blok B. Deze lange dagen werden stukken aangenamer gemaakt door de collega's die ik daar had. Natacha, ik wil je bedanken

voor de vele hulp die ik van je gekregen heb tijdens het uitvoeren van de experimenten. Door jouw hulp en gezelschap ging alles toch net dat tikkeltje sneller en vlotter. Félix en Wouter, bedankt voor de middagpauzes en de vele interessante gesprekken over HIPEC. Ik wens jullie beiden veel succes in het afwerken van jullie doctoraat en met jullie chirurgische carrière. Als één van jullie ooit een apothekersmening nodig heeft dan weten jullie mij te vinden!

De vele collega's die ik de afgelopen jaren op het labo gehad heb, wil ik bedanken voor de aangename werksfeer, de steun, de hulp, de wetenschappelijke discussies maar ook de gezellige middag- en koffiepauzes, de leuke laboactiviteiten & de teambuilding (extra dank voor Wesley&Lien L) Anouk, Bart, Lien L, Valerie, Jurgen, Lien D, Liesbeth, Ann-Katrien, Joke, Maxim, Bernd, Wesley, Marijke, Nane, Benoit en Kaat, ik wens jullie allen veel succes in jullie doctoraat en/of jullie verdere carrière. Een speciaal woordje van dank wil ik nog schenken aan mijn bureaugenoten. Bart, bedankt voor de vele grappige en geniale ideeën, gebruik deze genialiteit in je verdere carrière! Anouk, ook al vond ik je in het begin een "rare", ik kon mij geen betere buur en tennispartner voorstellen! Lien L, we delen dezelfde interesse en hebben eenzelfde doel voor ogen, ik hoop dat we binnen een paar jaar opnieuw kunnen samenwerken!

Wim, bedankt om mij alle technieken met het nodige geduld aan te leren. Katharine, Ilse en Christine wil ik bedanken voor de vele hulp bij alle administratie, voor alle kleine zaken mocht ik jullie komen storen en werd ik steeds met een lach en een aangename babbel geholpen. Daniël wil ik graag bedanken voor de interesse in mijn project en de hulp bij de in vivo experimenten tijdens het Itra-project.

Ook wil ik mijn thesisstudenten, Eline en Joke, bedanken voor hun hulp, inzet, interesse en enthousiasme tijdens hun onderzoekstage.

Als jonge sporter leerde ik dat ontspanning even belangrijk is als de inspanning die je levert om tot een goed resultaat te komen. Daarom wil ik iedereen bedanken die er de voorbijgaande jaren was om samen te gaan eten, samen iets te gaan drinken, op weekend te gaan,… In het bijzonder die mensen die er steeds zijn, Stefanie&Tony, Sophie&Bernard, Ellen, Elise, Ana&Wim, bedankt voor alle leuke momenten! Ook wil ik "mijn" zwemmers bedanken omdat ik mijn passie met hen kan blijven delen. Bert wil ik in het bijzonder bedanken voor het maken van de tekeningen.

Tenslotte mijn oprechte dank aan mijn familie, in het bijzonder mijn ouders en mijn broer, voor jullie steun, jullie liefde en de vele kansen die jullie me altijd geboden hebben. Jullie hebben me geleerd om altijd door te zetten, nooit op te geven en hebben me steeds gemotiveerd om altijd het beste uit mezelf naar boven te halen.

Als allerlaatste wil ik graag Laurent bedanken! Voor je onvoorwaardelijke steun, je liefde, je vertrouwen, je eindeloze geduld en je hulp wanneer ik het nodig had!

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LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
ANOVA	Analysis Of Variance
AUC	Area Under the Curve
AUC_{IP}	Area Under the peritoneal concentration-time Curve
AUC_{IV}	Area Under the plasma concentration-time Curve
CRC	Colorectal Cancer
CRS	Cytoreductive Surgery
DAB	3,3-Diaminobenzidine+
DACH	Diaminocyclohexane
DLS	Dynamic Light Scattering
DMSO	Dymethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSC	Differential Scanning Calorimetry
EPIC	Early Postoperative Intraperitoneal Chemotherapy
FIGO	International Federation of Gynaecological Oncologists
HIPEC	Hyperthermic Intraperitoneal Chemotherapy
HPLC	High Performance Liquid Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IP	Intraperitoneal
IV	Intravenous
LA-ICP-MS	Laser Ablation-Inductively Coupled Plasma-Mass Spectrometry
MRI	Magnetic Resonance Imaging
MTD	Maximum Tolerated Dose
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Difenyltetrazolium Bromide
NSCLC	Non-Small-Cell Lung Carcinoma
OC	Ovarian Cancer

PBS	Phosphate Buffered Saline
PC	Peritoneal Carcinomatosis
PCI	Peritoneal Cancer Index
PD	Pharmacodynamics
PI	Polydispersity Index
PK	Pharmacokinetic
PLGA	poly(lactic-co-glycolic)acid
Plu F127	Pluronic®F-127
PMP	Pseudomyxoma Peritoneï
Pt	Platinum
PTX	Paclitaxel
QC	Quality Control
QOL	Quality Of Life
RNA	Ribonucleic Acid
ROI	Region Of Interest
SAS	Statistical Analysis Software
SEM	Scanning Electron Microscope
SPSS	Statistical Program for Social Scientists
$T_{1/2}$	Half life
TGD	Tumor Growth Delay
UPLC	Ultra Performance Liquid Chromatography
UV	Ultraviolet
VOI	Volume of Interest

GENERAL INTRODUCTION AND THESIS OUTLINE

Ovarian cancer is the fifth most frequent cancer in females. 70% of the patients with ovarian cancer have advanced stage disease at the time of diagnosis as the spread of the cancer cells through the abdominal cavity occurs early in the development of the disease. Advanced stage ovarian cancer is characterized by metastatic deposits on the peritoneum called peritoneal carcinomatosis (PC). PC is responsible for a low quality of life and a high morbidity and mortality. Improvements in systemic chemotherapy have not resulted in a long-term survival. In the 1990s intraoperative intraperitoneal chemotherapy (IPEC) following a complete cytoreductive surgery (CRS) was developed for the treatment of PC replacing standard intravenous (IV) therapy.

IPEC has pharmacological and clinical advantages over IV chemotherapy in women with optimally debulked epithelial ovarian cancer. This alternative therapy is showing promising results with an increased 5-year survival of 44% compared to 30% after traditional IV treatment. The rationale for IP therapy is based on the presence of the peritoneal-plasma barrier which allows the use of higher cytotoxic doses in combination with a reduced systemic toxicity.

Although IPEC therapy has already been used for more than a decade, a lot of uncertainties still exist in terms of therapeutic schedule, used temperature, residence time, drug, or carrier solution.

In this study, paclitaxel (PTX) is used as chemotherapeutic agent as PTX has an appropriate pharmacokinetic profile for IP use. Due to its high molecular weight it has a limited absorption through the peritoneal-plasma barrier and when systemically absorbed its hepatic metabolism decreases the systemic side effects. Although PTX is a very promising molecule for IP treatment, it is not often used as allergic reactions are often observed due to the presence of Cremophor®EL, the vehicle of PTX in the commercially available formulation, Taxol®. Due to these toxicity issues a lot of research is performed to develop new PTX formulations in order to minimize the side effects. But all these developed formulations are not specifically

approved for IP therapy, and current IP chemotherapy in patients relies on the off-label use of products developed for IV applications.

Thesis outline

The **Introduction** provides a general overview of ovarian cancer, the development of peritoneal carcinomatosis and the main problems concerning the IPEC procedure. Moreover, in this introduction an overview can be found of different drug delivery systems developed for IP therapy.

In **Chapter 1** a new nanocrystalline PTX formulation with a high PTX-to-stabilizer ratio using the wet milling technique was developed which was suitable for IPEC treatment. In the same context, **Chapter 2** describes the evaluation of the new, commercially available PTX formulations, Abraxane® and Genexol®PM, which were compared with Taxol® to evaluate their suitability for IPEC treatment. For all formulations the toxicity was evaluated by determining the maximum tolerated dose (MTD), recovery time and bioavailability and the efficacy was evaluated by a tumor growth delay study (TGD) and by analysing the PTX tumor concentration. The toxicity and efficacy were evaluated in a xenograft rat model with peritoneal carcinomatosis of ovarian origin.

In most institutions hyperthermia is a standard component of the IPEC procedure although it remains unclear if the use of hyperthermia results in an beneficial effect. Therefore, in **Chapter 2** the additional effect of hyperthermia was evaluated both in vitro and in vivo in combination with the different PTX formulations.

As the limited drug penetration into tumor tissue is one of the major factors causing treatment failure, **Chapter 3** evaluates the PTX tumor penetration profile after IPEC treatment. On the basis of these results, the pharmacokinetic parameters such as the contact time and the PTX dose were evaluated.

Finally, in **Chapter 4**, a clinical study was performed in patients with peritoneal carcinomatosis of different origin. All patients underwent a cytoreductive surgery followed by an IPEC treatment with oxaliplatin, a frequently used chemotherapeutic agent. Patients were divided

in 3 treatment groups in order to evaluate the dose, the treatment time and the influence of hyperthermia on the efficacy and toxicity of the IPEC treatment.

INTRODUCTION

HIPEC AS TREATMENT FOR PERITONEAL CARCINOMATOSIS OF OVARIAN ORIGIN

Parts of this chapter are published in:

L. De Smet, W. Ceelen, J.P. Remon, C. Vervaet, Optimization of Drug Delivery Systems for Intraperitoneal Therapy to Extend the Residence Time of the Chemotherapeutic Agent, The Scientific World Journal, (2013), Article ID 720858, 7 pages, http://dx.doi.org/10.1155/2013/720858.

INTRODUCTION

With more than 40 000 new cases every year in Europe, ovarian cancer is the second most common gynecological malignancy [1] and it is responsible for 5% of all cancer deaths in women [2]. The stage of the disease at the time of initial diagnosis is an important determinant of ovarian cancer survival. Ovarian cancer is known as a silent killer, as the symptoms of ovarian cancer are vague such as abdominal pain, bleeding and abdominal swelling, so 70% of the patients have advanced stage disease (stage III or IV) at the time of diagnosis [3].

The different stages (Fig. 1) are characterized by:

- Stage I: The cancer is still contained within the ovary (or ovaries). The cancer has not spread outside the ovary.
- Stage II: The cancer is in one or both ovaries and has spread to other organs (such as the uterus, fallopian tubes, bladder, the sigmoid colon, or the rectum) within the pelvis. The cancer has not spread to lymph nodes, the lining of the abdomen (the peritoneum), or distant sites.
- Stage III: The cancer is in one or both ovaries, and the cancer has spread beyond the pelvis to the peritoneum and/or to the lymph nodes.
- Stage IV: This is the most advanced stage of ovarian cancer. In this stage the cancer has spread to the inside of the liver, the lungs, or other organs located outside the peritoneal cavity.

In the 1960s, the 5-year relative overall survival for ovarian cancer was 30%. Improvement of the quality of the cytoreductive surgery (CRS) as well as development of novel drugs and new chemotherapeutic regimens have increased this 5-year relative overall survival to 44%, with the rates being 92%, 72%, 27% and 21% for stage I to IV, respectively (SEER-website, 2013) [3].

Despite the advances made in cytotoxic therapy, the 5-year relative overall survival for patients with advanced stage disease remains low so it is important to understand the development of peritoneal carcinomatosis in order to respond better to the evolution of the cancer.

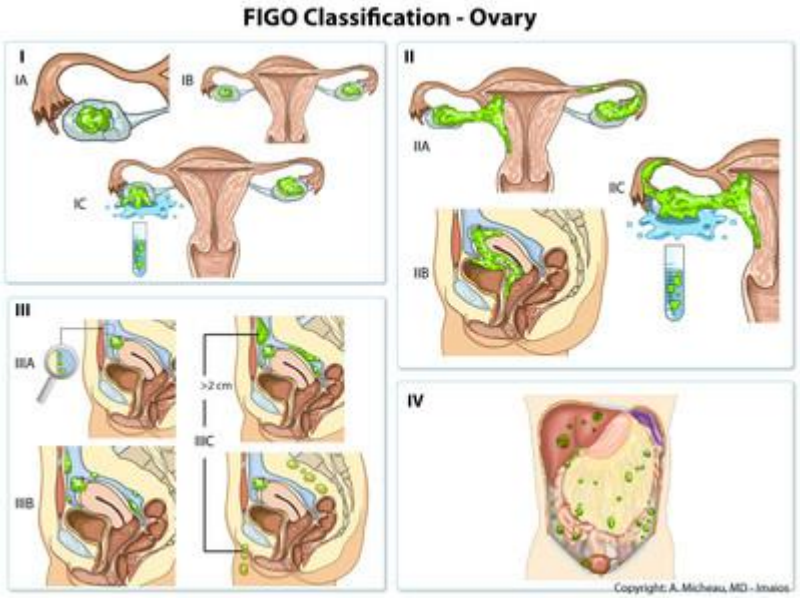

Figure 1. The FIGO classification (named after its authors, the International Federation of Gynaecological Oncologists) of the different stages of ovarian cancer.

PHYSIOPATHOLOGY OF PERITONEAL CARCINOMATOSIS

At the early stage of ovarian cancer the tumor is limited to one or both ovaries. The most common type of ovarian cancer, serous ovarian cancer, arises directly from the cells of the ovarian surface epithelium [4]. As a consequence of tumor growth, the capsule becomes disrupted and the tumor spreads beyond the confines of the ovaries by direct extension and invasion of adjacent organs such as the uterus, the mesothelial lining and the peritoneum [5]. Some malignant cells will detach from the original tumor. These exfoliated malignant tumor cells can be transported throughout the abdominal cavity by the peritoneal fluid before seeding intraperitoneally (Fig. 2).

Diaphragmatic respiratory movements and intestinal peristalsis result in hydrostatic pressure differences between the lower and upper abdomen which are capable of conveying peritoneal fluid from the pelvis to the subhepatic and subphrenic regions, even in the upright position. This transport can affect multiple vital organs within the abdomen, including the gastrointestinal systems. Nests of tumor cells are commonly observed on the omentum, the mesentery and the diaphragm [6].

This seeding is also associated with the formation of malignant ascites, resulting in raised intra-abdominal pressure, abdominal distention and discomfort. In contrast to most other cancer types, dissemination through the vasculature is rare [7]. However in advanced stage disease, there is a high incidence of pelvic and paraaortic lymph node involvement [8, 9].

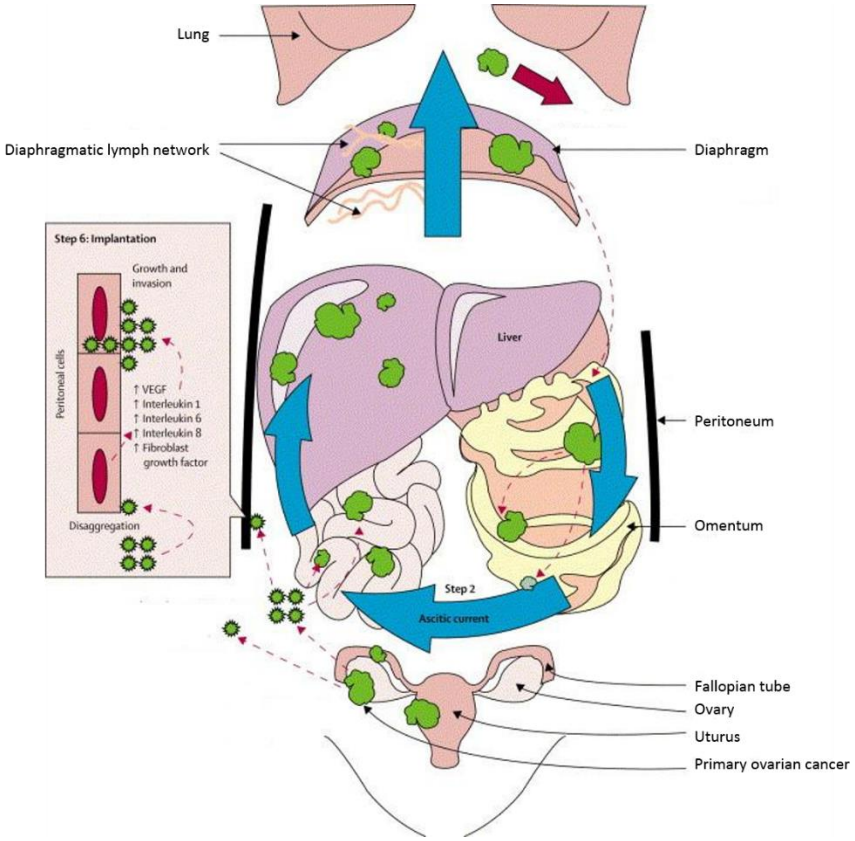

Figure 2. The mechanism of peritoneal dissemination from ovarian origin (adapted from [7]).

TREATMENT OF ADVANCED OVARIAN CANCER

Peritoneal carcinomatosis (PC) is associated with gastrointestinal symptoms and impaired quality of life (QOL), and is often a marker of poor prognosis. In the final stages of PC, patients suffer from severe anorexia, dyspnea and pain from malignant bowel obstruction, ascites and pleural effusion as a result of extensive tumor burden [10]. PC of ovarian origin was traditionally regarded as a terminal condition. The terminal nature of this disease has been demonstrated in a large clinical trial, the French EVOCAPE I trial, which prospectively followed patients with PC of different primary cancers from diagnosis till death. The overall survival for patients with PC of ovarian origin was 12 to 23 months [11].

In the early 1990s, adjuvant intravenous (IV) chemotherapy with cisplatin or carboplatin in combination with cyclophosphamide was the standard of care for the treatment of advanced ovarian cancer. Since the mid-90s cytoreductive surgery (CRS) followed by a combination IV therapy with a platinum compound and a taxane became the first line treatment [12, 13]. Despite this progress in systemic chemotherapy, most patients relapsed and ultimately died from their disease. The addition of intraperitoneal (IP) chemotherapy for the treatment of advanced ovarian cancer has been showing promising results.

Since the 1980s, different treatment options for patients with isolated PC were proposed based on the hypothesis that PC is a locoregional disease which would benefit from a local therapeutic approach [14]. The rationale of using local IP therapy for the treatment of PC is based on the pharmacokinetic advantages, such as high local concentration with a longer half-life of the drug in the peritoneal cavity, which improves the interaction of the cytotoxic agents with the cancer cells.

INTRAPERITONEAL CHEMOTHERAPY

The current treatment protocols for peritoneal carcinomatosis are based on a combination of cytoreductive surgery (CRS) and perioperative intraperitoneal chemotherapy.

The aim of CRS is to eliminate all macroscopic disease. At the beginning of the surgical procedure, most surgeons quantify the extent of the peritoneal carcinomatosis by the Peritoneal Cancer Index (PCI) [15]. The PCI gives valuable information about the exact distribution of seeding and tumor volume, representing in detail the extent of the peritoneal spread. The PCI is an assessment that combines lesion sizes (0 – 5 cm) with tumor distribution (abdominal-pelvic regions 0 – 12). The extent of the disease is quantified as a numerical score (fig. 3). The PCI score has been found to be an prognostic indicator of survival.

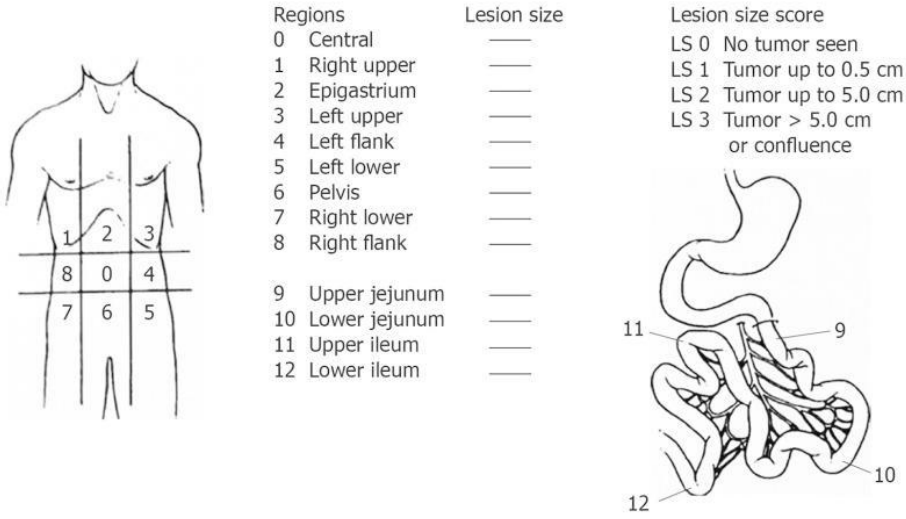

Figure 3. The peritoneal cancer index.

After CRS the residual tumor nodules are also quantified and scored as the size of the residual tumor nodules is the major prognostic indicator for the outcome of the disease. The definition of complete CRS is no visible evidence of cancer (CC-0) or only small residual nodules with a size less than 2.5 mm (CC-1), since this ensures sufficient drug penetration into the tumor during subsequent intraperitoneal chemotherapy. CRS with residual tumors with a diameter >2.5 mm (CC-2 and CC-3) is considered to be incomplete and these patients have a low survival rate [16, 17].

A second essential part of the current management of PC is the perioperative intraperitoneal chemotherapy which targets the residual microscopic disease. IP treatment of patients with PC was first reported by Spratt et al., who used triethylenethiophosphoramide in a patient with pseudomyxoma peritonei (PMP) [14] and by Speyer & Myers, who administered 5-Fluorouracil (5-FU) and methotextrate intraperitoneally under normothermic conditions to 16 patients with PC [18]. In 1988, Koga et al. reported intraperitoneal chemotherapy in 23 patients with PC from gastric cancer origin [19].

The adjuvant perioperative intraperitoneal chemotherapy can be applied directly after CRS under normothermic or hyperthermic conditions (i.e. (hyperthermic) intraperitoneal chemotherapy or (H)IPEC) as a part of the surgical procedure, or can be started the first postoperative day and continued for several (usually five) days (i.e. early postoperative intraperitoneal chemotherapy or EPIC). No randomized trials have been published comparing

the survival outcomes between EPIC and HIPEC [20]. EPIC has been associated with a higher risk of complications after CRS, possibly due to the prolonged contact between the chemotherapeutic agent and damaged tissues at the operated surfaces [21]. Other disadvantages of EPIC are an uneven distribution of the chemotherapeutic fluid in the abdomen and less comfort for the patients (e.g. nausea and impaired mobility) linked to the chemotherapeutic fluid present in the abdomen [22]. As the main drawback of (H)IPEC is the necessity to perform this technique in the operating room, requiring specialized equipment and an experienced perfusionist, (H)IPEC is the preferred technique.

PHARMACOKINETIC RATIONALE BEHIND IP THERAPY

The rationale behind the IP administration of chemotherapy is based on the presence of a peritoneal-plasma barrier [23]. The peritoneum consists of 2 parts which line the peritoneal cavity, the visceral peritoneum covers the intra-abdominal organs and mesenteries while the parietal peritoneum lines the abdominal wall, the pelvis, the anterior surfaces of the retroperitoneal organs and the inferior surface of the diaphragm. The total area of the peritoneum can be compared to the skin area.

The peritoneum barrier is a complex, three-dimensional structure made up of (a) the peritoneum, a monolayer of mesothelial cells supported by a basement membrane, (b) five layers of connective tissue which include interstitial cells, a collagen matrix, hyaluronan and proteoglycans and (c) the cellular component which consists of fibroblasts, pericytes, parenchymal cells and blood capillaries. Contrary to intuition, it is not the mesothelial lining which is the main transport barrier but the capillary walls and the surrounding interstitium which are the most important barriers for the transport from the abdominal cavity to the plasma [24].

In general there are 3 exits from the peritoneal cavity: (1) diffusion through the parietal peritoneal surfaces, (2) diffusion through visceral peritoneal surfaces, and (3) absorption through lymphatics.

The rate at which a drug leaves the peritoneal cavity and enters the plasma is the critical mechanism by which peritoneal drug concentrations fall following IP administration.

The transport through the peritoneum was described by a simplified mathematical formula, where both plasma and the peritoneal cavity are considered as a single compartment separated from each other by an effective membrane [25].

$$\text{Rate mass transfer} = PA\ (C_p - C_B)$$

With PA the permeability area (effective contact area x permeability), C_p the concentration in the abdominal cavity and C_B the concentration in the blood. This formula indicates the importance of the size of the effective contact area of the peritoneal membrane.

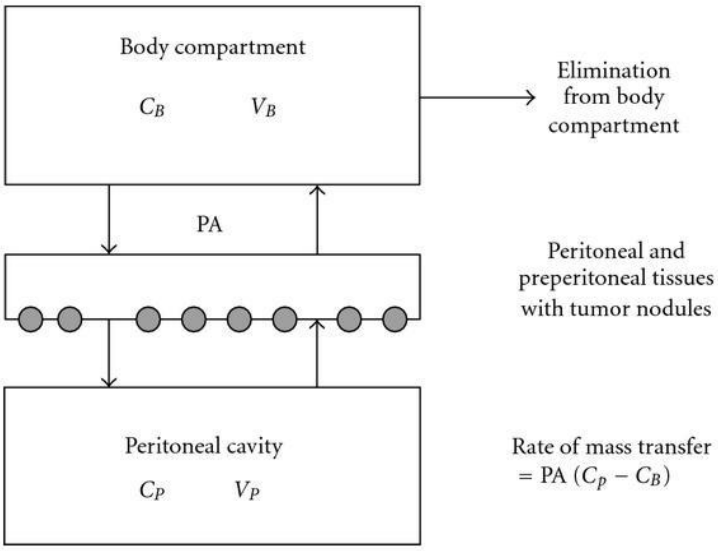

Figure 4. Traditional two-compartment model of peritoneal transport in which transport of a drug from the peritoneal cavity to the blood occurs across the peritoneal membrane.

This model (Fig. 4) indicates that large molecular weight substances such as many chemotherapeutic agents would be cleared more slowly from the peritoneal cavity than from the systemic circulation. This would increase drug exposure to the tumor-containing area. The pharmacokinetic rationale of (H)IPEC is based on the dose intensification provided by the administration of chemotherapy into the peritoneal cavity and the delayed clearance caused by the peritoneal plasma barrier. The peritoneal clearance is inversely proportional to the

square root of the molecular weight of the drug which results in a higher concentration in the peritoneal cavity than in the plasma after intraperitoneal administration [26].

This model suggests furthermore high local drug concentrations which are in contact with the tumor-affected region but this leads not automatically to high tumor concentrations. This model does not reveal anything about the specific tumor penetration. A high concentration gradient as driving force may lead to an increased diffusion into the tumor. Many studies have shown only a limited tissue and tumor penetration of the chemotherapeutic agents. This limited penetration may limit antitumor effect, but it also may protect sensitive normal cells on the mucosal side of the gastrointestinal tract.

POINTS TO CONSIDER ABOUT (H)IPEC

It has been found that IP therapy and in particular (H)IPEC is a promising therapy for PC treatment. Although IP therapy is already conducted for many years in different clinical institutions, no standard treatment in terms of schedule, residence time, drug, or carrier solution has been established. Parameters such as open or closed technique, chemotherapeutic agent, temperature, perfusate volume and duration of the perfusion differ from institution to institution. Hereafter issues as the technique, the use of the cytostatic drug, the available pharmaceutical formulations and the used temperatures are discussed.

1. (H)IPEC, the technique

(H)IPEC can be performed by the open, the partially closed and the closed technique. Whether an open or (partially) closed technique is used, is based on the surgeon's preference [27]. When performing the open (H)IPEC technique, the procedure is carried out before closing the abdominal cavity. The open abdomen is covered with a plastic sheet, to create a "Coliseum" container (Fig. 5). The plastic sheet is opened in the midline to allow the surgeon to manipulate the viscera and a smoke evacuator is placed to clear aerosolized chemotherapy. By manipulation, the surgeon is causing a homogenous distribution of the chemotherapeutic agent in the abdominal cavity. Cancer cells from surfaces that would otherwise not be accessible are therefore reached [15].

Figure 5. Hyperthermic intraperitoneal chemotherapy administered using the open technique.

Environmental safety aspects are an argument for the use of the closed (H)IPEC technique. During this procedure the abdomen is closed before administration of the chemotherapy. There are two variations of closed techniques. In the first one, only the skin is closed. After (H)IPEC the abdomen is reopened, and the surgeon will perform the anastomoses. This technique is called the partially closed technique. For the closed technique, the anastomoses were performed before closing the abdominal cavity and administration of the chemotherapy. Studies already have proven that thermal homogeneity and a homogenous distribution of the chemotherapy were less obtained during the closed techniques [28].

2. Drug selection for IP chemotherapy

In the case of IP therapy, the cytotoxic agent must be able to penetrate the peritoneal surface and the tumor nodules effectively, in combination with its ability to eradicate microscopic residual disease within the peritoneal fluid. During IP therapy, drug delivery to peritoneal tumors is twofold. The primary route is drug diffusion through the tumor interstitium, while the second route is the recirculation of the drug absorbed from the peritoneal cavity (Fig. 6). However, it is evident that the latter route is of minor importance as efficient and safe IP therapy requires systemic drug levels as low as possible in order to minimize systemic side effects caused by the cytotoxic agent.

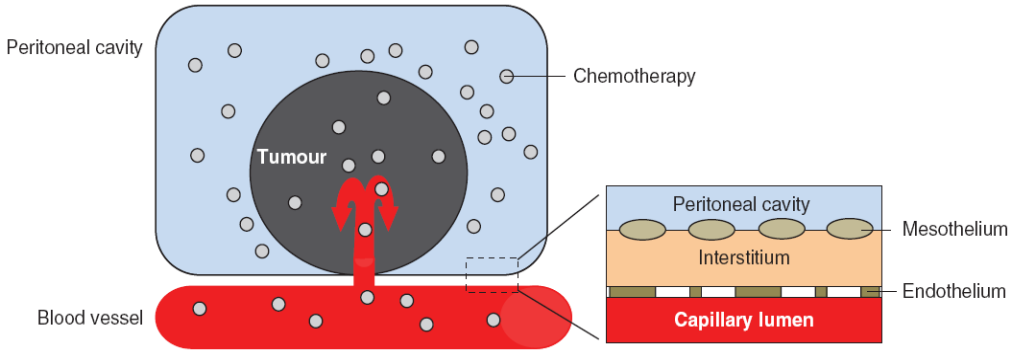

Figure 6. Chemotherapeutics enter the tumor through direct surface contact and by crossing the peritoneal-plasma barrier and entering the tumor microcirculation.

One of the major challenges of IP therapy is to maintain a high local drug concentration within the peritoneal cavity to provide a sufficient concentration gradient as driving force for drug diffusion into the tumor. The residence time of small molecular weight drugs in the peritoneal cavity may not be sufficient because they are quickly absorbed through the peritoneal capillaries into the systemic circulation [29]. Therefore, the drug delivery system used for IP therapy is an important factor to deliver drugs efficiently to the target tissue. In addition, different parameters must be considered to select the ideal chemotherapeutic agent for IP therapy and to maximize its efficacy: cavity-to-plasma AUC ratio, systemic absorption, depth of tumor penetration, intrinsic activity of the agent against the primary tumor type [30].

In general, water insoluble molecules with a high molecular weight and a high peritoneal-to-plasma AUC ratio remain longer in the peritoneal cavity and are thus preferred for IP treatment (Table I) [23, 31]. One important consideration is that a high peritoneal-to-plasma AUC ratio does not automatically confer a higher efficacy, since the penetration of the chemotherapeutic agent into the tumor might be limited.

Table I. Water solubility, the partition coefficient (log P) and peritoneal-to-plasma drug area under the curve (AUC) ratio of intraperitoneal chemotherapeutic agents.

Chemotherapeutic agent	Molecular weight	Water solubility	Log P	Peritoneal-to-plasma AUC ratio
Cisplatin	300	Good	-2.19	12
Carboplatin	371	Good	1.06	10 – 18
Oxaliplatin	397	Good	1.73	16
Paclitaxel	854	Poor	3.54	1000
Docetaxel	862	Poor	2.92	181
5-Fluorouracil	130	Sparingly	-0.89	367
Doxorubicin	544	Poor	1.27	474

The ideal drug for IP therapy has a high penetration into the tumor nodule. This high penetration in combination with a slow diffusion of the chemotherapeutic solution through the capillary endothelium results in low systemic concentrations and a reduced systemic toxicity by rapid metabolism of the drug.

Paclitaxel

In the early 1960s, the cytostatic effect of the Taxus Brevifolia was shown through a program of the National Cancer Institute (USA). 10 years later in 1971, the active compound paclitaxel was isolated and characterized, and another 10 years later the method of action was discovered [32, 33]. They found that PTX binds to the microtubule rather than to the tubulin dimers, both essential for mitosis, maintenance of cell shape, intracellular transport, cell signaling and division. The β-tubulin in the microtubule is the cellular target for PTX. PTX forms very stable and dysfunctional microtubules and is thus known as a microtubule-stabilizing agent. Hereby, PTX blocks the normal microtubule dynamics during mitosis. This causes that the cell goes into a mitotic arrest followed by apoptosis and necrosis [34].

The use of PTX for IP treatment of PC of ovarian origin is rational, because of its high activity against ovarian cancer cells and expected favorable pharmacokinetics because of its limited

absorption from the peritoneal cavity due to its high molecular weight (853.9), low water solubility (20 µg/ml) and significant first pass effect.

Different studies demonstrated a significant pharmacokinetic advantage of PTX for IP therapy. The first study of PTX IP therapy was published by Markman et al. (1994) [35]. The AUC after IP treatment was 1000-fold higher compared to the plasma AUC, and persisted for more than 24 – 48 hours after a single dose. Other studies [36] showed significant peritoneal concentrations till one week after IP administration. PTX proved already its excellent activity against ovarian cancer since the 1990s, with a low toxicity and good tolerance in peritoneal administration. Experimental studies have demonstrated that even short-time exposure of tumor cells to high concentrations of PTX, as during HIPEC, are extremely sufficient to induce extended cell growth arrest and cell death by necrosis and apoptosis [37, 38].

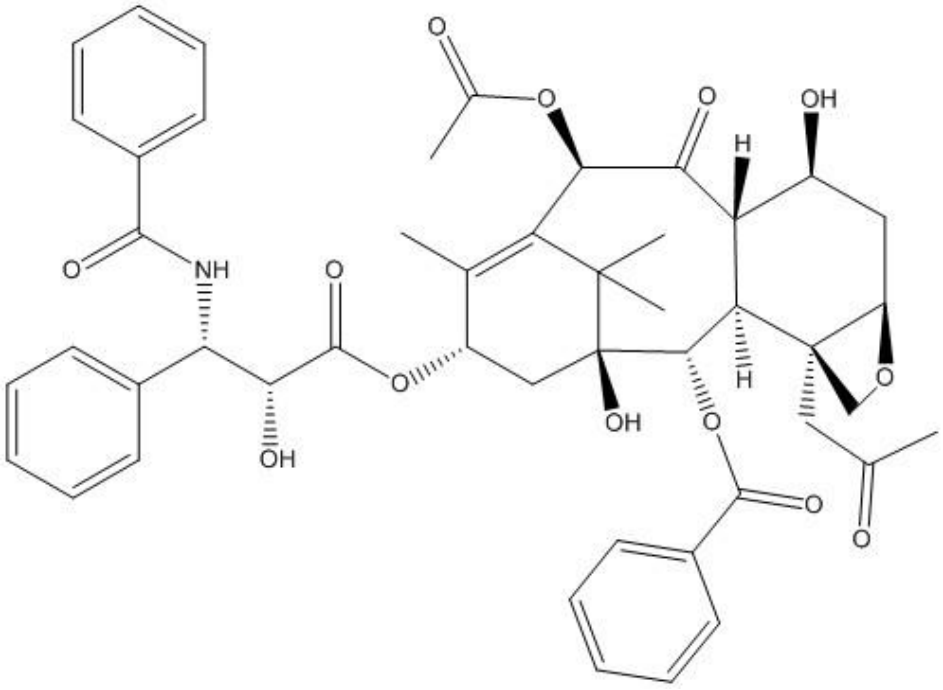

Figure 7. Chemical structure of paclitaxel.

Although PTX is a very promising molecule for IP therapy, it is not often used in clinical practice as it causes various side effects, as serious dose-limiting toxicities such as myelosuppression, peripheral sensory neuropathy and allergic reactions. In 40% of the patients hypersensitivity reactions are observed and 3% of the patients have potentially life-threatening side effects. These side effects have been associated with Cremophor®EL, present in the first commercially

available formulation Taxol® (i.e. paclitaxel dissolved in a 50/50 (v/v) mixture of ethanol and Cremophor®EL). In recent years a lot of research was performed to develop new PTX formulations on the one hand and on the other side to discover novel second-generation taxanes both with the goal to increase the selectivity and efficacy, and to reduce the systemic toxicity. Table II gives an overview of the new taxane analogs (Table IIa) and new PTX formulations (Table IIb) which are FDA-approved or in the final stage of clinical development for the treatment of various solid tumor malignancies [39].

Table IIa: New second-generation taxanes.

Analogues	Potential indications
Cabazitaxel	Prostate [40] Breast [41]
Tesetaxel	Breast [42] Non-small-cell lung carcinoma [43] Gastroesophageal [44] Melanoma
Larotaxel	Non-small-cell lung carcinoma [45] Breast [46]

All these formulations are very promising but are developed for IV treatment. Today there are no chemotherapeutic formulations specifically approved for IP therapy, and current IP chemotherapy in patients relies on the off-label use of products developed for IV applications.

These formulations designed for IV therapy often suffer from a rapid clearance from the peritoneal cavity, fast absorption through the lymphatics, no tumor selectivity and local and systemic toxicity caused by the high blood concentrations after systemic absorption. These issues, which reduce the efficacy of IP therapy, dramatically limit the use of conventional IV chemotherapeutic formulations for IP use [47]. Inadequate drug delivery to solid tumors is a major cause of IP treatment failure. As a result, a number of drug delivery strategies (which are reviewed in the remainder of this Introduction) have been investigated to extend the residence time of the chemotherapeutic agent and to optimize IP therapy (Table III).

Table IIb. New paclitaxel formulations.

Name	Composition	Potential indications
Abraxane®	Albumin-bound nanoparticles of PTX	Breast [48] Ovarian [49] NSCLC [50] Ovarian [53] Pancreas [51]
Opaxio®	Polyglumex-CT2103 Prodrug Water-soluble polymer of glutaric acid linked to PTX	NSCLC [52] Ovarian [53] Glioblastoma Esophageal [54]
Genexol®PM	Polymeric-micellar (biodegradable block copolymer which includes monomethoxy poly(ethylene glycol)-block-poly(D,L lactide))	NSCLC [55] Breast [56] Gastric [57]
Taxoprexin®	Docosahexaenoic acid (DHA)-PTX Prodrug	NSCLC [58] Melanoma

Oxaliplatin

The successful treatment of a variety of cancers with metal-containing anti-cancer drugs started with cisplatin [59]. Although the compound was already described in 1845, its anti-cancer activity was only discovered in 1964 [60]. Due to the side effects of cisplatin, a second generation platinum-containing agent, carboplatin, was developed, and over the past 30 years these compounds have dominated the anticancer treatments. In addition to the side effects, the use of (cis)platinum was also hampered by the fact that certain tumors developed resistance against it. Hence intensive research programmes were initiated to develop less toxic and resistance-free platinum complexes [61]. As a result more than 3000 platinum analogues were synthetized over the past 30 years. However, only 12 reached clinical trials and currently just 3 platinum-compounds are registered on the European market (cisplatin, carboplatin and oxaliplatin).

Oxaliplatin (oxalate (trans l-1,2-diaminocyclohexane)platinum)) (Fig. 8), a third generation platinum-containing agent, is developed to improve the toxicological profiles and overcome the platinum-resistance in solid tumors. In an oxaliplatin molecule, both amino groups of cisplatin are replaced by a diaminocyclohexane group (DACH) while an oxalate group is added as leaving group to improve the solubility. These groups play an important role in the cytotoxic effect of oxaliplatin [62, 63].

The method of action of oxaliplatin starts with a non-enzymatic transformation by the displacement of the oxalate group which appears responsible for the formation of its ultimate cytotoxic form. The inert DACH-complex enters the cell and causes DNA damage. Apoptosis of cancer cells can be caused by formation of DNA lesions, arrest of DNA synthesis, inhibition of RNA synthesis, and triggering of immunologic reactions [63, 64]. Oxaliplatin also has a synergestic effect in combination with 5-FU but the underlying method of action is not well understood [65].

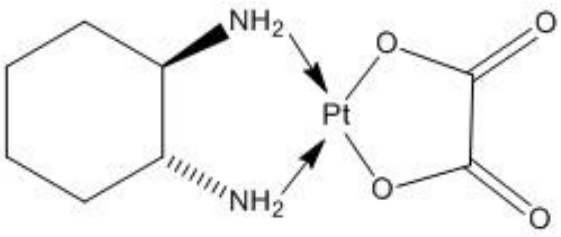

Figure 8. Chemical structure of oxaliplatin.

Oxaliplatin was already discovered in 1976 but was only introduced in clinical trials in 1985 [66]. It was used for the first time for the treatment of advanced colon cancer in the mid-90s in combination with 5-FU. Oxaliplatin (Eloxatin®) was EMA approved (France) in 1996 and FDA approved in 2002 for the treatment of advanced and metastatic colon cancer in combination with 5-FU but it is active against a variety of cancers including ovarian and gastric cancer and malignant mesothelioma [67-69].

Oxaliplatin has provided significant response rates alone or in combination with 5-FU and leucovorin for the treatment of advanced stage colon cancer [63]. After intravenous oxaliplatin administration, the penetrated concentration into the tumor tissue is not sufficient to eliminate the disease effectively. For PC, experimental studies have shown that

intraperitoneal oxaliplatin treatment improved the exposure of oxaliplatin to the peritoneal surfaces compared to intravenous therapy [70]. The choice for oxaliplatin for IPEC treatment is rational as it has favourable pharmacokinetic and pharmacodynamic properties [71]. Elias et al. has shown that IPEC treatment with oxaliplatin in combination with intravenous 5-FU improved the overall survival [72] without increasing the systemic toxicity [73]. For ovarian cancer, IPEC with oxaliplatin is mainly used for patients showing resistance against cisplatin or hypersensitivity reactions [74].

3. Drug delivery systems for IP therapy

Microspheres

Microspheres (>1 µm) can be designed to release the drug gradually over time using a wide variety of biodegradable and biocompatible polymeric substances. The size of the microspheres is the most important factor that influences residence time in the peritoneum, since microspheres smaller than 8 µm may disappear from the peritoneal cavity through the lymphatic capillaries [75].

Microspheres for IP drug delivery are often based on biodegradable aliphatic polyesters of hydroxyl acids such as poly(lactic-*co*-glycolic)acid (PLGA) and related polymers. PLGA was for example used to incorporate cisplatin (*cis*-dichlorodiammmine-platinum(II)) in microspheres. Cisplatin is a hydrophilic molecule which is, in solution, rapidly absorbed from the peritoneal cavity by the capillaries and transferred to the systemic circulation. Therefore biodegradable anti-cancer drug-loaded microspheres were developed which showed a long retention and sustained release of the drug. Cisplatin was released from the PLGA matrix by diffusion until 14 days after IP administration. During this period the particles were retained in the abdominal cavity for a long period and gradually absorbed, which reduced the systemic side effects [76]. Another drug delivery system using PLGA is paclitaxel-loaded microparticles [77]. This system consists of two types of paclitaxel-loaded microparticles with different drug release rates obtained by the lactide/glycolide (L/G) ratio. First, a burst release was observed (L/G 50:50), followed by a sustained drug release (L/G 75:25). As already described PTX is a promising molecule for IP treatment, so PTX-containing microsphere formulations based on other

polymers were also developed. Paclimer® was made by incorporating PTX in a biodegradable poly-phosphoester polymer matrix, to form microspheres with a mean particle size of 53 µm. These microparticles resulted both in vitro and in vivo (after IP administration in a phase I study) in a sustained release of PTX over a period of 8 weeks [78]. The triblock poly (ε-caprolactone)-poly(ethyleneglycol)-poly (ε-caprolactone) (PCL-PEG-PCL) copolymer was synthesized to prepare camptothecin-loaded microspheres. These microspheres were developed to protect camptothecin from hydrolysis, thus enhancing its treatment efficacy towards colorectal PC [79].

Nanoparticles

Although microspheres have a longer retention time in the peritoneal cavity, they can also induce inflammatory reactions and peritoneal adhesions [80]. Because of these issues the benefit-to-risk ratio should be taken into account when deciding between microparticles and nanoparticles. Kohane et al. showed that nanoparticles and microparticles formulated with lower molecular weight polymers had a much lower incidence of peritoneal adhesions and were safer to use [81]. Another advantage of nanoparticles is that they can bypass drug efflux pumps, thus evading multi-drug resistance and achieving significantly higher drug accumulation in the cells compared to IP therapy with unformulated free drugs [82] [83]. Although conventional nanoparticles are rapidly cleared from the abdominal cavity due to their size, nanoparticles which respond to a wide array of stimuli such as pH, temperature, light and ultrasound, are being investigated. For IP therapy, paclitaxel-loaded pH responsive nanoparticles were developed which were designed to deliver paclitaxel intracellularly after endocytosis. In this formulation PTX is encapsulated in an acrylate-based polymer with a pH-responsive 2,4,6-trimethoxybenzaldehyde protective group. These nanoparticles react to an endosomal pH (pH ≤5) and increase in volume to release their drug load. When these nanoparticles are IP injected in a mice tumor model, they remain in the peritoneal cavity for 7 days [84]. IP chemotherapy using a nanocrystalline PTX formulation stabilized by Pluronic®F-127 (i.e. polyethylene oxide-polypropylene oxide (PEO-PPO) block copolymer) showed a faster recovery of the animals compared to treatment with Taxol®, while the cytotoxicity and antitumor efficacy was similar [85]. In 2008 a phase I study for the IP treatment of PC of ovarian origin using Nanotax® was initiated, using PTX nanoparticles produced by a technique

known as precipitation with compressed antisolvent. In vivo studies showed that mice treated with Nanotax® survived significantly longer and demonstrated a reduced toxicity compared to the Taxol®-treated group [86].

Liposomes

Liposomes have been widely studied as potential carriers for hydrophilic and hydrophobic drugs and diagnostic agents. Due to their small size (100 – 1000 nm), liposomes have a fast clearance from the abdominal cavity. As such it is important to change some of the liposomes characteristics such as lipid composition, surface properties and charge, in order to increase their retention time in the abdominal cavity. Hirano et al. (1985) described that the charge of the liposomes is a predictive factor for the retention time [29]. When the liposomes have a negative charge, they were rapidly absorbed from the peritoneal cavity, while positively charged liposomes had a slower absorption rate. This might be attributed to electrostatic interaction between the positively charged liposomes and the negative surface of the peritoneal mesothelium, in combination with a low uptake of positive liposomes by peritoneal macrophages [87]. Changing the type of phospholipid (the main building block of liposomes) had no effect on retention time in the abdominal cavity [88], whereas the incorporation of polyethylenglycol (PEG) in the phospolipid membrane showed a 30% higher peritoneal retention by the avoidance of the macrophages present in the peritoneal cavity, compared to the same non-pegylated liposomes [87].

Micelles

Taxol®, a micellar PTX formulation using Cremophor®EL (i.e. a polyethoxylated castor oil surfactant) is used for IP treatment of ovarian cancer. Taxol® showed a longer residence time in the abdominal cavity compared to free unformulated PTX (40.7 ± 13.8 hours vs 7.3 ± 2.8 hours), which indicated that encapsulation is an effective way to extend the residence time of a drug in the abdominal cavity [89]. But as already described, Taxol® is not well tolerated as hypersensitivity reactions and neurotoxicity are reported due to the surfactant (Cremophor®EL) included in the formulation [90]. Several studies showed that the carrier plays an important factor in the distribution and clearance of the drugs after IP administration [89] [91]. In a rat model, the absorption rate of PTX and docetaxel was influenced by the

solubilizer in the system (Cremophor®EL and polysorbate-80 for paclitaxel and docetaxel, respectively). While the AUC_{IP} of PTX doubled in comparison to docetaxel when conventional vehicles were used, dissolution of docetaxel in Cremophor®EL or Polysorbate-80 increased its retention time and yielded similar AUC_{IP} values compared to PTX [92].

Table III. Advantages and disadvantages of the drug delivery systems investigated for IP therapy.

Drug Delivery system	Advantages	Disadvantages
Microspheres	Prolonged retention	Limited tumor penetration Risk of peritoneal adhesions
Nanoparticles	Passive targeting Avoiding multidrug resistance (MDR) Lower incidence of peritoneal adhesions	Rapid clearance from abdominal cavity
Liposomes	Similar to nanoparticles Active targeting by varying parameters	Similar to nanoparticles
Micelles	Prolonged retention time	Increasing systemic toxicity
Injectable systems	Prolonged retention time Localized and sustained drug delivery Lower systemic toxicity Prevention against peritoneal adhesion	Viscosity issues
Implantable systems	Similar to injectable systems	Invasive Surgical expertise

Implants and injectable depots

Implantable and injectable depots have been investigated for localized and sustained delivery of anticancer agents [93]. Those systems may be the most promising approach for the IP treatment of PC. The rationale of using hydrogels for IP therapy is dual as a hydrogel will retain the drugs within the peritoneum and will also protect against peritoneal adhesions [94]. A major drawback for hydrogels is achieving homogeneous distribution of the drug in the peritoneal cavity. Also the viscosity of the injectable hydrogels can cause problems. Low viscous sytems may fail to provide a delayed drug release profile, while highly viscous systems may be difficult to administer [93]. Because of these problems thermosensitive hydrogels are developed. These gels are free-flowing at room temperature and form a non-flowing gel at body temperature, serving as an in situ drug depot. Therefore the formulation can be easily mixed with the drugs and injected by a syringe as a fluid, while forming a viscous deposition at the target location [95]. This gelation behavior is primarily due to the formation of self-associated micelles via hydrophobic interactions. Above the lower critical solution temperature, these micelles are closely packed together resulting in micellar aggregation and a change of rheological properties [96]. Gong et al. reported that a hydrogel system based on a biodegradable poly(ethylene glycol)-poly(ε-caprolactone)-poly(ethylene glycol) (PEG-PCL-PEG, PECE) triblock copolymer could be used as delivery vector of chemotherapeutic drugs for IP infusion chemotherapy. The system was loaded with 5-FU [97] and doxorubicin [98]. When the system was infused intraperitoneally in mice, the formed gel phase guaranteed a delayed drug release up to 48 hours after injection.

Implantable systems were developed for IP treatment of ovarian cancer. This implant is composed of paclitaxel-loaded poly-D,L-lactide and poly(lactide)-block-poly(ethylene glycol) (PLA-b-PEG) particles dispersed throughout a chitosan egg-phophatidylcholine matrix. This formulation provided a sustained and localized release of 1% PTX per day in mice over a period of 3 months [99, 100]. The implants have a higher efficacy, and are less toxic and more biocompatible than Taxol®. Although the implantable systems have promising results, the biggest issue for using those systems is the need for surgical expertise to implant the system.

Carrier solutions

A simple approach to improve IP administration of chemotherapeutic agents using conventional formulations is varying the medium in which the drug is dissolved or suspended. The volume of the fluid in which the chemotherapeutic agent is dissolved plays an important role towards drug distribution in the peritoneal cavity. The ideal carrier solution for IP chemotherapy should expose all cancerous surfaces or residual tumor cells to high levels of cytotoxic agents for as long as possible and ensure a uniform distribution of the drug in the abdominal cavity. Small volumes of fluid do not flow freely in the peritoneum, even with multiple position changes of the patients [101], while large volumes (>2 L/m^2 body surface area) which cause moderate abdominal distention result in more uniform intraperitoneal drug distribution. Hence maintaining a high volume of the intraperitoneal liquid would improve the effectiveness of the treatment as the choice of the solution, in which the drug is administered, plays an important role in the distribution of the drugs [102] [103]. Current techniques for IP chemotherapy administration mainly use isotonic electrolyte solutions (e.g. 5% glucose, 0.9% sodium chloride). However isotonic salt and dextrose solutions are rapidly absorbed due to their low molecular weight [103, 104] [102] which decreases the distribution of the drug in the peritoneal cavity. Hypotonic solutions showed promising in vitro results as they increased cytotoxicity and accumulation of cisplatin in tumor cells [105]. However, the clinical results were negative as bleeding and thrombocytopenia occurred, while no pharmacokinetic advantages were observed [106] [107]. When hypertonic solutions were used, a prolonged retention time of the intraperitoneal volume was achieved, but their main disadvantage is the dilution of the intraperitoneal drug due to fluid shift to the peritoneal cavity [102]. 4% icodextrin, a colloid osmotic agent of α-1,4 linked glucose polymers, has been successfully used to prolong retention of intraperitoneal chemotherapy, the solution can remain 3 to 4 days in the peritoneum [108]. Another iso-osmolar solution, with a potentially long intraperitoneal dwell time, is 6% hydroxyethyl starch (hetastarch) which has been successfully used to prolong intraperitoneal retention of gemcitabine and paclitaxel in animal studies [102, 109]. Also the use of HAES-steril®, a starch-based carrier solution, reduced the clearance of the chemotherapeutic solutions from the peritoneal cavity compared to physiological saline solution [110].

4. Rationale of hyperthermia

As already described intraperitoneal chemotherapy is a promising technique for the treatment of PC. In most institutions hyperthermia is a standard component of the surgical procedures of the treatment. Hyperthermia is defined as an increase of temperature in a tumor-affected body region to 39 – 43 °C by using an external energy source [111]. The efficacy of hyperthermia is shown in different in vitro studies. In the 1960s it was thought that heat acts similarly to radiation by directly damaging nuclear DNA [112]. In 1986, Borrelli et al. described the occurrence of “membrane blebbing” which is a typical feature of apoptosis [113]. Currently several mechanism are known which argument the efficacy of hyperthermia:

- Cytoskelet
    - Changes in stability and fluidity of cell membrane
    - Alterations of cell shape
    - Impaired transmembrane transport
    - Alteration of membrane potential
    - Modulation of efflux pumps
    - Induction of apoptosis
- Intracellular proteins
    - Impairment of protein synthesis
    - Denaturation of proteins
    - Aggregation of proteins at nuclear matrix
    - Induction of HSP-synthesis
- Nucleic acids
    - Decrease of RNA/DNA synthesis
    - Inhibition of RNA/DNA enzymes
    - Altered DNA-conformation
- Other alteration of cell function
    - Intracellular metabolism of other substrates
    - Gene expression, signal transduction

Another observation was that hyperthermia not only acts in a cytotoxic way in itself but also sensitizes tumor cells to radiotherapy (thermal radiosensitization) and various cytostatic drugs (thermal chemosensitization). Conflicting results have been reported from in vitro and in vivo studies on the combination of PTX with hyperthermia (Table IV).

Table IV. Impact of hyperthermia on paclitaxel activity.

In vitro			
Study	Temperature (°C)	Thermal enhancement	Cell type
Michalakis [37]	41.5 – 43	-	Ovarian adenocarcinoma (SKOV-3)
Mohammed [109]	41.5	+	Murine fibrosarcoma (Fsa-II)
Othman [114]	43	+	Murine breast cancer (FM3A)
Knox [115]	42	-	Human cytotoxic T-lymphocytes
Rietbroek [116]	42 – 43	-	Human squamous lung cancer
Leal [117]	43	-	Human breast adenocarcinoma (MCF-7)
Van Bree [118]	41	-	Human colorectal cancer
In vivo			
Sharma [119]	43	+	Murine melanoma cells (B16F10)
Cividalli [120]	43	+	Murine mammary carcinoma (CH3/TIF)
Mohamed [109]	41.5	-	Murine fibrosarcoma (Fsa-II)
Bouquet [121]	43	-	Colorectal cancer (CC531s)

The rationale for the use of hyperthermia with taxanes is based on the fact that mild hyperthermia resulted in a disorganization of the microtubule system, and taxanes are considered microtubule stabilizing agents. Thus, a common target of cytotoxic damage is involved. Mohamed et al. (2003) showed that when PTX was combined with hyperthermia, there was a statistically significant decrease in tumor growth at low doses when compared with PTX alone [109]. Michalakis et al. showed that cell death mechanisms were different after hyperthermic treatment as a significantly higher amount of cell necrosis was observed compared to the normothermic treated cells. Despite the increased cell necrosis, the number of viable cells remained unchanged [37, 38]. Nevertheless, they suggest that a combination of

PTX and hyperthermia supported clinical relevance as the induced necrosis increased local inflammation and recruited resources of the immune system.

In vitro and in vivo results often differed, with factors such as tumor physiology, microcirculation, pH and hypoxia playing an essential role in the interaction between hyperthermia and different cytotoxic agents. Major investigators on this issue have stated that drug concentrations at the target must be high to allow sufficient thermal enhancement. These conditions are met during HIPEC. But other studies have shown that the use of HIPEC can cause side effects such as bone marrow suppression, renal failure, anastomic leakage and bowel perforation. Anastomic leakage and bowel perforation are related with the applied temperature, the risk of damage increased at higher temperature (>43 °C) or when there is long contact time between heat and tissue.

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CHAPTER 1

DEVELOPMENT OF A NANOCRYSTALLINE PACLITAXEL FORMULATION FOR HIPEC TREATMENT

Parts of this chapter are published in:

L. De Smet, P. Colin, W. Ceelen, M. Bracke, J. Van Bocxlaer, J.P. Remon and C. Vervaet, Development of a nanocrystalline Paclitaxel formulation for HIPEC treatment, Pharmaceutical Research, 29 (9), 2398-2406 (2012).

INTRODUCTION

Ovarian cancer is the fifth most common cancer affecting European women [1]. Once the tumor starts growing in the ovary, spread of cancer cells throughout the abdominal-pelvic cavity occurs very early in the development of the disease [2]. The standard therapy for patients with peritoneal carcinomatosis of ovarian origin is initial cytoreductive surgery followed by intravenous platinum-taxane chemotherapy [3, 4]. Lately, this standard therapy has been modified as alternative treatments have been developed. A treatment following cytoreductive surgery is hyperthermic intraperitoneal chemotherapy (HIPEC) is a new treatment strategy with promising results. Administration of chemotherapy intraperitoneally under hyperthermic conditions may improve the mean overall survival of patients with advanced ovarian cancer from 19.0 to 76.1 months [5]. PTX is a suitable molecule for HIPEC treatment as it has a high peritoneal/plasma concentration ratio (>1000) and a significant first pass effect [6]. However, PTX is not commonly used for HIPEC treatment because of the side effects caused by Cremophor®EL used as solubilizer in the commercially available formulation Taxol®, like abdominal pain and life-threatening hypersensitivity reactions [7]. Due to these side effects a lot of research is done, developing new PTX formulations without resorting to toxic excipients to improve the solubility of PTX.

As size reduction is an efficient method to improve the performance of poorly soluble drugs (the increased surface area of smaller particles enhances dissolution rate and bioavailability based on the Noyes-Whitney equation), wet milling was used to obtain a nanosuspension of paclitaxel. While both top-down (particle size reduction) and bottom-up (precipitation method) techniques can be used to manufacture nanoparticles [8], wet milling is a typical top-down method using milling beads to grind the particles. This procedure is known as an efficient method to prepare nanoparticles with easy scale-up and limited batch-to-batch variability when the method is optimized. Contamination of the final product due to erosion of the milling beads is a main concern, while changes in physical form or amorphization can also be an issue during wet milling [9]. As breakage of drug crystals into nanoparticles significantly increases particle surface area, the higher Gibbs free energy creates a thermodynamically unstable nanosuspension, and proper selection of a stabilizer is required during the

preparation of the nanosuspension to prevent agglomeration or crystal growth (due to Ostwald ripening) of the nanoparticles [10].

In this study, the wet milling technique is applied to obtain a PTX nanosuspension stabilized with a surfactant (polyethylene oxide-polypropylene oxide block copolymers, Pluronic®F-68 and
Pluronic®F-127). Pluronic-stabilized PTX nanocrystals have already been formulated, but were characterized by a low drug-to-stabilizer ratio, Lui et al. required at least a PTX/Pluronic®F-127 ratio of 1/5, as at lower stabilizer concentrations stable nanocrystals could not be formed [11]. In order to maximize the drug concentration at the delivery site, which is one of the challenges of HIPEC therapy [12], the stabilizer content in the nanocrystalline formulation was minimized in our study. After characterization of the nanocrystals processed via the wet milling technique, the feasibility of the nanosuspension for HIPEC treatment was assessed (in comparison with Taxol®) by evaluating in vitro cytotoxicity of the excipients as well as the formulation on an ovarian cancer cell line, while the toxicity, bioavailability and effect on tumor growth were tested in a rat model.

MATERIALS AND METHODS

Materials

Paclitaxel (PTX) was purchased from Enzo Life Sciences (Zandhoven, Belgium). Polyethylene oxide-polypropylene oxide (PEO-PPO) block copolymers, Pluronic®F-68 and Pluronic®F-127, were obtained from BASF (Ludwigshafen, Germany), Taxol® from Bristol-Myers Squibb (Brussels, Belgium) and Cremophor®EL from Alpha Pharma (Waregem, Belgium).

Preparation of paclitaxel nanocrystals

PTX nanosuspensions were prepared by a wet milling technique using two different stabilizers (Pluronic®F-68 and Pluronic®F-127) in three PTX/stabilizer ratios (2/1, 4/1 and 8/1). After dissolving the stabilizer in a 20 ml vial containing 5 ml of 0.9% NaCl, PTX powder (50 or 100 mg) was dispersed in this aqueous phase. Zirconium oxide beads (amount 30 g, diameter 0.5

mm) were added to the suspension as milling pearls. The vials were placed on a roller-mill (Peira, Beerse, Belgium) and grinding was performed at 150 rpm for 24 or 60 hours. After milling, the nanoparticles were separated from the grinding pearls by sieving.

For solid state characterization of the PTX nanocrystals, the nanosuspension was freeze dried for 24 hours at -50 °C and 1 mbar.

Nanocrystal characterization

The mean particle size and polydispersity index (PI) of the nanosuspensions was determined by photon correlation spectroscopy, using a Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). Prior to analysis, the nanosuspensions were diluted with distilled water and were analysed at room temperature.

The morphology of the freeze dried drug particles was observed under a scanning electron microscope (SEM) (JSM 5600 LV, Jeol, Tokyo, Japan) after coating the powder particles with platinum using a sputtering equipment (Auto Fine Coater, JFC-1300, Jeol, Tokyo, Japan).

Thermal properties of the freeze dried samples were analysed by differential scanning calorimetry (DSC Q2000, TA instruments, Leatherhead, UK). Samples were placed in sealed aluminum pans, and evaluated over a temperature range from -20 to 190 °C with a heating rate of 10 °C/min. Pure drugs and physical mixtures were tested as controls. The thermal profiles were analysed using TA Instruments Universal Software.

In vitro cytotoxicity

The human ovarian carcinoma cell line (SKOV-3, obtained from the American Type Culture Collection) was cultured at 37 °C in a 5% CO_2-containing humidified atmosphere in McCoys medium (Invitrogen, Merelbeke, Belgium). The medium was supplemented with 10% fetal bovine serum, penicillin, streptomycin (Invitrogen, Merelbeke, Belgium) and fungizone (Bristol Myers Squibb, Brussels, Belgium).

The cytotoxicity of PTX nanosuspensions (at a PTX/Plu F127 ratio of 4/1) and Taxol® was tested at PTX concentrations of 0.01, 0.1, 1, 5 and 10 µg/ml and 8 wells per concentration were used.

Both Taxol® and PTX nanosuspensions were diluted with 0.9% NaCl to the appropriate concentration. In addition, the cytotoxicity of the excipients in these formulations (Pluronic®F-127 and Cremophor®EL) was tested using 9 concentrations: 0, 0.01, 0.1, 1, 1.5, 2, 2.5, 3 and 3.5 mg/ml.

To evaluate the cytotoxicity, $20x10^3$ cells/ml were seeded in 96-well plates (Sarstedt, Newton NC, USA). After 72 hours, 20 µl medium was removed and replaced by the test formulation. After incubation for 1 hour at 41.5 °C (i.e. to mimic the HIPEC procedure used during in vivo studies), the medium was entirely removed, cells were washed with phosphate buffered saline (PBS) and 200 µl fresh medium was placed in each well. Afterwards, the cells were incubated for 24 and 96 hours at 37 °C under 5% CO_2-atmosphere. The cytotoxicity of the test formulations was determined via MTT assay and compared with the non-treated cells. 100 µl medium was replaced by 100 µl MTT-reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-difenyl-tetrazolium bromide, at a concentration of 1 mg/ml PBS-D^+). The reagent was mixed and incubated in dark conditions for 2 hours at 37 °C. Afterwards all medium was removed and 200 µl DMSO (Acros Organics, Geel, Belgium) was added to dissolve the formed formazan. After incubation of the plates for 1 hour at 37 °C, the optical density was measured at 570 nm normalizing with a reference wavelength of 650 nm using an ELISA-plate reader (Paradigm Detection Platform, Beckman Coulter, Suarleé, Belgium).

In vivo testing

HIPEC procedure

Adult female athymic nude rats (Harlan, Horst, The Netherlands) were kept in standard housing conditions with water and food ad libitum and a 12 hours light/dark circle. All animal experiments were approved by the Ethical Committee of the Faculty of Medicine, Ghent University (ECD 09/06).

After anesthetizing each rat with 3% isoflurane (Forene®, Abbott, Waver, Belgium) a vertical incision was made along the midline in the abdominal wall muscle. The abdominal wall muscle was attached to a metal ring which was placed a few centimeters above the incision. The inlet and outlet tubing (Pumpsil®, Watson-Marlow, Zwijnaarde, Belgium) was placed in the

peritoneal cavity for perfusion with the cytostatic solution over a period of 45 min. A roller pump (Watson-Marlow, Zwijnaarde, Belgium) circulated the cytostatic solution through a heat exchanger set at 41.5 °C. During perfusion, the perfusate solution and body temperature of the rat were closely monitored and data was collected using E-Val® 2.10 Software (ELLAB®, Roedovre, Denmark). After HIPEC, the cytostatic perfusate solution was removed and the incision was sutured.

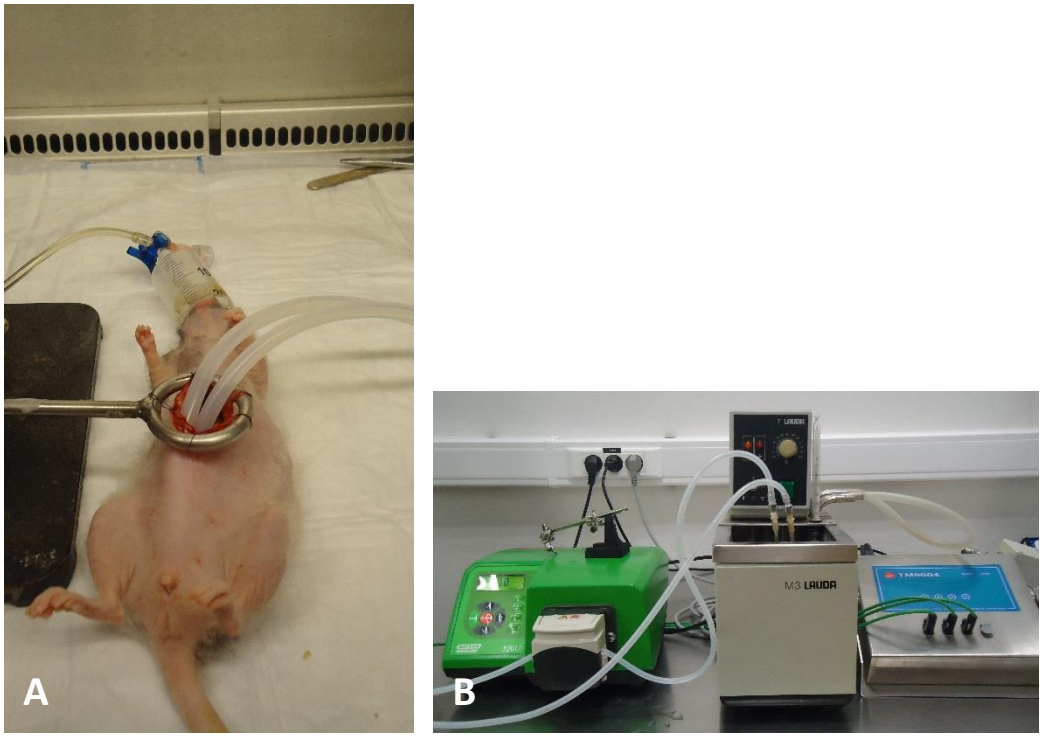

Figure 1. Athymic nude rat which underwent a HIPEC procedure (A) and the equipment for the HIPEC procedure, from the left to the right, the roller pump, heat exchanger and temperature sensor (B).

Maximum tolerated dose

The maximum tolerated dose (MTD) was determined for both PTX formulations (Taxol® and PTX/Plu F127 nanosuspension (ratio 4/1)). The MTD was defined as the highest non-lethal dose with a maximum reduction of body weight of 10% after 2 weeks of HIPEC treatment. The test procedure was based on the Organization for Economic Co-operation and Development (OECD) guidelines. Based on the MTD of Taxol® (0.24 mg/ml) determined by Bouquet et al. in WAG/Rij rats [13], a PTX dose of 0.21 mg/ml was used as starting point. To determine MTD, the PTX concentration in the formulation was gradually increased (increments of 0.03 mg/ml) using 1 rat per concentration. When mortality occurred, 3 more rats were tested at the highest

non-lethal dose to confirm MTD. MTD of both formulations was determined using HIPEC settings (i.e. 41.5 °C during 45 min). The PTX nanosuspensions as well as Taxol® were diluted with 0.9% NaCl to obtain the correct dose in an isotonic solution.

Bioavailability

Blood was sampled in heparin-containing tubes via a catheter which was placed in the arteria carotis. Blood samples were taken at 0, 15, 30, 45, 60 and 90 min after starting the perfusion. Perfusate samples were collected at 0, 15, 30 and 45 min after starting the perfusion and the PTX concentration was measured via HPLC, to ensure that an accurate dose was administered during the HIPEC procedure. After the bioavailability study the rats were euthanized. Blood samples were centrifuged immediately afterwards, and separated plasma was stored frozen at -20 °C until analysis.

The perfusate samples were analysed by high performance liquid chromatography (HPLC). The HPLC-system (Merck-Hitachi, Tokyo, Japan) consisted of a pump (L-6000), an integrator (D-2000), an autosampler (L-7200) with a 25 µl loop and a UV/VIS detector (L-4200). Detection was performed at 227 nm. To achieve chromatographic separation a guard column (Lichrospher® 100-RP-18, 4*4 mm (5 µm), Merck, Darmstadt, Germany) and an analytical column (Lichrospher® 100-RP-18, 125*4 mm (5 µm), Merck, Darmstadt, Germany) were used. The mobile phase consisted of acetonitrile (Biosolve, Valkenswaard, The Netherlands) and 0.1% (v/v) phosphoric acid in ultrapure water (Acros Organics, Geel, Belgium) (42:58, v/v) degassed by ultrasonication under vacuum.

Frozen plasma samples, calibrator samples and quality control (QC) samples were allowed to thaw at room temperature. For samples within the assay range a volume of 50 µl was transferred to an empty well of a 96-well filtration plate. Other samples (samples above upper limit of quantitation) were diluted sixfold with blank rat plasma prior to the analysis. Subsequently 200 µl of acetonitrile containing internal standard (C^{13}-paclitaxel) was added. After vacuum filtration, the filtrate was diluted with 150 µl of water and injected onto the Waters Acquity UPLC system for analysis. After chromatographic separation on a Waters Acquity UPLC BEH C18 column (100 mm x 2.1 mm, 1.7 µm particle size), PTX and the internal standard were detected using a Waters Quattro Ultima triple quadrupole system (Micromass

Waters, Manchester, UK). The validation of the method was conducted with reference to the FDA's guidance for bioanalytical method validation. The calibration curve was constructed by least squares linear regression of the peak area ratio of PTX/internal standard against nominal concentration with a weighting of concentration^{-1}. The measurement range of the analytical method was 2.0 – 500 ng/mL for PTX in rat plasma. The range was further extended up to 3 µg/ml by appropriately diluting plasma samples prior to analysis. Total imprecision and trueness were calculated on results of repeated analysis of quality controls on different days. For all levels of the QC samples, imprecision and trueness measurements comply with the FDA guidance specifications on maximum tolerable bias and imprecision.

Tumor growth delay

Donor rats were injected with 30x10^6 SKOV-3 cells between the peritoneum and the abdominal muscle. The animals received daily subcutaneous cyclosporine injections (dose: 3 mg) over a period from 3 days prior until 10 days after tumor cell injection. After 3 to 4 weeks, a tumor was induced, but this tumor remained localized and did not (or not completely) penetrate the peritoneum. Therefore parts of the tumor induced in the donor rats (5 x 5 mm, with a thickness of 3 mm) were transplanted on the peritoneum of acceptor rats. The acceptor rat also received daily subcutaneous cyclosporine injections (dose: 3 mg) from 3 days prior until 10 days after tumor transplantation to ensure tumor attachment. Two weeks after transplantation, the tumor had attached to the peritoneum and had sufficiently grown to perform the tumor growth delay (TGD) experiment.

The effect of the PTX formulations (Taxol® and nanosuspension) on tumor growth was evaluated via a Siemens® Trio 3T MRI (Erlangen, Germany). Prior to the MRI scan, the rats were anaesthetized with Rompun 2% (Bayer, Diegem, Belgium) and ketamine 1000 CEVA (Ceva, Amersham, UK) using a dose of 10 mg/kg and 90 mg/kg, respectively. The rats were placed prone in a (wrist) coil to measure the tumor volume. A T1-weighted 3D FLASH sequence was applied with a flip angle of 10°, a repetition time of 13 ms and an echo time of 4.9 ms to obtain a voxel size of 0.19 x 0.19 x 0.4 mm^3. In order to easily locate the tumor, the rat was palpated and a vitamin B12 pellet was attached to the skin where the tumor was located. Tumor volume was calculated using PMOD software (PMOD Technologies, Adliswil, Switserland). Rats were scanned 1 day before HIPEC treatment (day 0) to measure the initial

volume of the tumor. At day 1 the rats were treated with Taxol® or the nanocrystalline PTX formulation. Tumor volume was evaluated 7 and 14 days after HIPEC treatment to monitor the effect of both PTX formulations.

Statistical analysis

Statistical Program for Social Scientists (SPSS 19.0) was used to analyse the results.

For the bioavailability study, the pharmacokinetic parameters of both groups were compared using an unpaired sample *t*-test with a significance level of 0.05.

For the TGD study, data of day 0 were used as reference (100%). The different treatment groups were compared with each other on day 7 and 14 using a one-way ANOVA with a significance level of 0.05. Bonferroni post-hoc analysis was performed for pairwise comparisons between treatment groups.

RESULTS AND DISCUSSION

Physico-chemical characterization of PTX nanocrystals

Pluronic®F-68 and Pluronic®F-127 were selected as stabilizers because these block copolymers have already been successfully used to stabilize PTX nanosuspensions (both at a higher surfactant/PTX ratio than used in this study) [11]. In addition, these surfactants are known to increase the solubility of low soluble drugs, and have cytotoxicity-promoting properties as they interact with multi-drug resistance cancer tumors, resulting in drastic sensitization of these tumors to the cytostatic drugs [14-16]. A wet milling cycle during 24 hours did not yield nanocrystalline PTX when Pluronic®F-68 was used as stabilizer (independent of the PTX/stabilizer ratio) (Table I).

Table I. Mean particle size ± SD (nm) and polydispersity index after wet milling (at 150 rpm) of a paclitaxel suspension.

Stabilizer	PTX/Stabilizer ratio (w/w)	Amount PTX (mg)	Milling time (hours)	Size ± SD (nm)	PI
Pluronic®F-68	2/1	100	24	4057 ± 1042	0.368
	4/1	100	24	3374 ± 1731	0.421
	8/1	100	24	3208 ± 765	0.470
Pluronic®F-127	2/1	100	24	417 ± 72	0.351
	4/1	100	24	462 ± 128	0.308
	8/1	100	24	812 ± 154	0.425
	2/1	100	60	420 ± 18	0.260
	4/1	100	60	440 ± 30	0.268
	8/1	100	60	462 ± 66	0.302
	2/1	50	60	325 ± 12	0.224
	4/1	50	60	307 ± 12	0.232
	8/1	50	60	375 ± 21	0.287

In contrast, Pluronic®F-127-containing formulations were efficiently ground into nanosuspensions. Although Pluronic®F-68 and F-127 have the same basic PEO-PPO-PEO structure, they differ in the number of PEO and PPO groups. The higher molecular weight and lower hydrophilic-lipophilic balance (HLB) value of Pluronic®F-127 compared to Pluronic®F-68 allowed more interaction between the amphiphilic surfactant and the nanoparticle surface, providing sufficient steric hindrance to stabilize the nanoparticles and prevent particle agglomeration [17]. However, a minimum concentration of stabilizer was required as at the lowest Pluronic®F-127 content (i.e. PTX/stabilizer ratio of 8/1) the surfactant failed to sufficiently stabilize the PTX particles. A longer milling time (60 hours) not only yielded PTX nanocrystals at all PTX/Pluronic®F-127 ratios, it also resulted in a narrower particle size distribution as indicated by the lower polydispersity indices (PI).

Reducing the PTX amount during wet milling to 50 mg improved the efficiency of the milling process as all formulations yielded a mean particle size below 400 nm, in combination with

analysis showed a significant reduction in particle size compared to unmilled PTX (>5 µm), as a size around 400 nm was obtained, which confirms the results obtained by particle size analysis (Fig. 2).

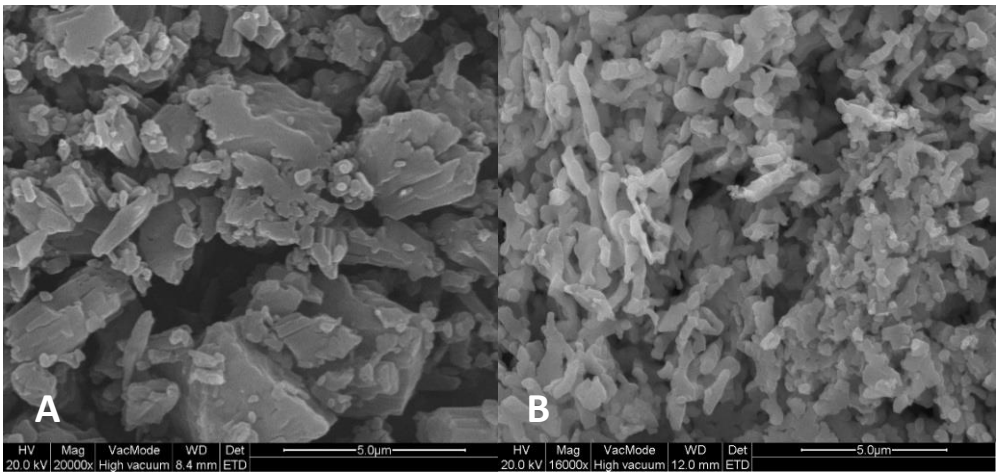

Figure 2. SEM images of (A) unmilled paclitaxel and (B) freeze dried paclitaxel nanocrystals, processed via wet milling (150 rpm for 60 hours), using a paclitaxel load of 50 mg per vial and a PTX/stabilizer ratio of 4/1. Pluronic®F-127 was used as stabilizer.

Solid state characterization by DSC of the freeze dried nanocrystalline formulation showed that the crystallinity of PTX was not affected by the wet milling process (Fig. 3). As no crystalline-to-amorphous transitions were observed, the friction generated during the wet milling process did not create stability issues [18].

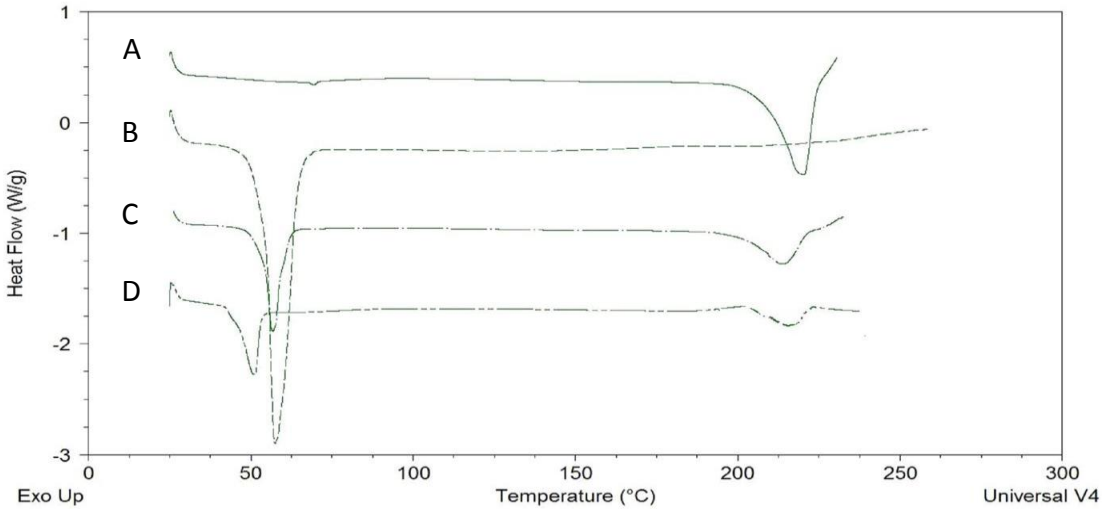

Figure 3. Differential scanning calorimetry profiles of paclitaxel (A), Pluronic®F-127 (B), physical mixture (C) and PTX/Plu F127 4/1 nanocrystals (D).

A 6 month stability study at ambient conditions of the different PTX/Plu F127 formulations (Fig. 4) indicated that initially the particle size of all 3 formulations slightly increased. Afterwards the particle size of PTX/Plu F127 2/1 and 4/1 nanocrystals remained constant (± 400 nm) with PI values staying below 0.3. In contrast, the PTX/Plu F127 8/1 formulations became polydispers (PI>0.5) and particle agglomeration was observed. Due to these stability issues the PTX/Plu F127 8/1 formulations was not used in further experiments. As the goal of this study was to reduce the stabilizer concentration in the nanosuspensions, the PTX/Plu F127 4/1 formulation was selected for further in vitro and in vivo experiments.

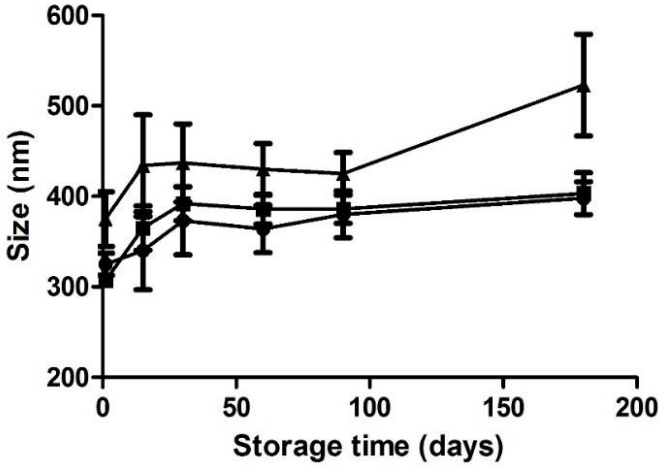

Figure 4. Particle size (mean ± SD) of PTX nanosuspensions as a function of storage time at ambient conditions. PTX/Plu F127 ratio: (●) 2/1 , (■) 4/1, (▲) 8/1

In vitro cytotoxicity

The cytotoxicity of the PTX formulations (Taxol® and the nanosuspension) as well as the cytotoxicity of the excipients (Pluronic®F-127 and Cremophor®EL) was tested on human ovarian carcinoma cells (SKOV-3) as ovarian cancer commonly results in PC which can be treated via HIPEC. As the main drawback to the use of Taxol® for HIPEC treatment are the side effects caused by its excipient Cremophor®EL, the cytotoxicity of Cremophor®EL and Pluronic®F-127 was compared in a concentration range from 0.01 to 3.5 mg/ml. After 1 hour incubation, there was no reduction of cell viability at the lowest concentration (Fig. 5).

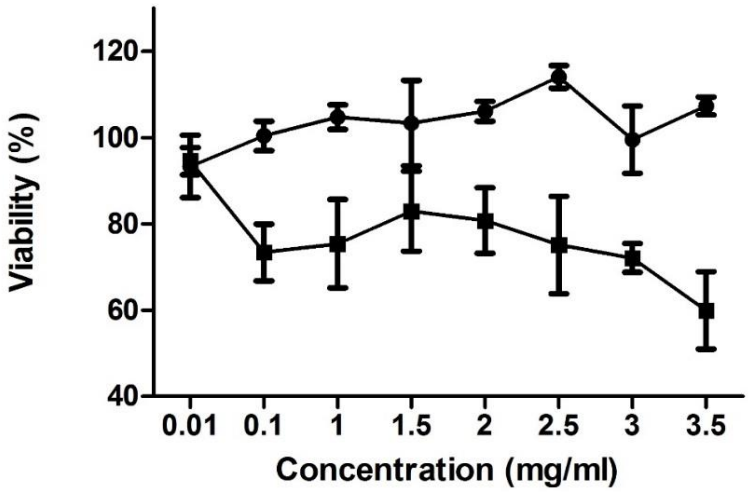

Figure 5. Viability (mean ± SD) of the SKOV-3 cell line after application of different concentrations of Cremophor®EL (■) and Pluronic®F-127 (●) at hyperthermic conditions (41.5 °C) (n=8 wells per concentration).

However, at higher concentrations the cell viability decreased after contact with Cremophor®EL while the cells treated with Pluronic®F-127 remained unaffected, indicating a significantly lower cytotoxicity of Pluronic®F-127 compared to Cremophor®EL. Despite the differences between the two excipients, both PTX formulations were equipotent, as the cytotoxicity of the new nanosuspension was equal to Taxol® (Fig. 6).

Nanocrystalline PTX formulation for HIPEC treatment

The maximum tolerated dose (MTD) of both PTX formulations after HIPEC was determined by monitoring the survival rate and body weight of the rats following HIPEC treatment. While at a PTX dose of 0.21 and 0.24 mg/ml the rats regained their initial body weight after 2 weeks, a PTX concentration of 0.27 mg/ml for Taxol® and the nanosuspension resulted in mortality. Hence, 0.24 mg/ml was set as MTD for both formulations. At this concentration no significant differences were observed between both treatments based on the weight of the rats 2 weeks after treatment. However, rats treated with the PTX nanosuspension recovered faster compared to the group treated with Taxol® as they already regained their initial body weight 5 days after HIPEC treatment, highlighting the advantage of using the non-cytotoxic

Pluronic®F-127. Based on the body surface area, the MTD corresponded to a dose of 960 mg/m^2, which is much higher compared to the dose administered to humans (175 mg/m^2) during HIPEC [19]. This underlines one of the opportunities of HIPEC: the possibility to use higher doses, resulting in higher local concentrations which are maintained for a longer time in the abdominal cavity and which have a higher direct cytotoxic effect [20].

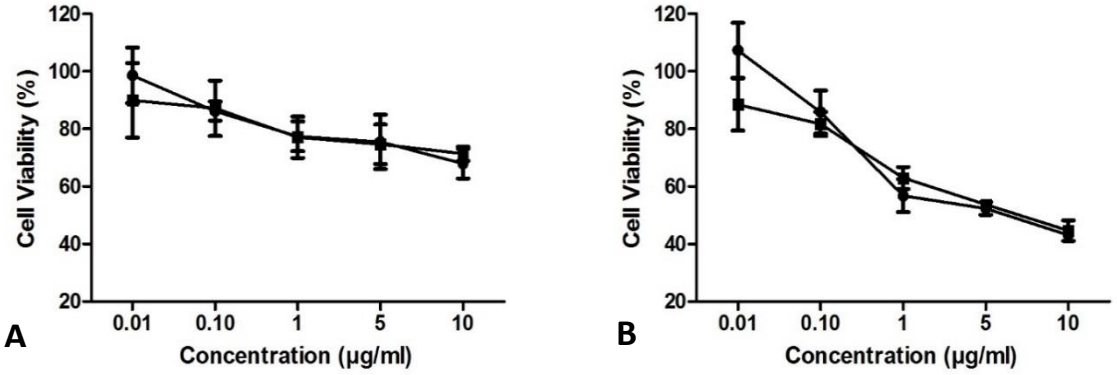

Figure 6. Viability (mean ± SD) of the SKOV-3 cell line (n=3 and 8 wells per concentration) after application of different paclitaxel concentrations delivered under hyperthermic conditions (41.5 °C) via (■) Taxol® and (●) PTX/Plu F127 4/1 nanosuspension. MTT after 24 hours (A) and 96 hours (B).

During HIPEC treatment, a sample of the perfusate was taken every 15 min in order to monitor the delivered PTX concentration. Statistical analysis showed no differences between the applied concentration of the different formulations (p=0.348).

Monitoring the PTX plasma concentrations over a 90 min period (Fig. 7) showed similar concentrations for Taxol® and the PTX nanosuspension during the perfusion period. However, when the cytotoxic agent was removed after HIPEC treatment (i.e. after 45 min) the PTX plasma concentrations of the nanosuspension increased, while PTX plasma levels after Taxol® treatment remained constant during the entire monitoring period (i.e. 90 min). The enhanced absorption of PTX was also reflected in the pharmacokinetic parameters after perfusion with the PTX nanosuspension: in comparison to Taxol®, C_{max} was significantly higher (124.7 ng/ml vs. 42.0 ng/ml, p=0.03), and $AUC_{Pl,\ t=\ 90\ min}$ was 1.5-fold higher but not significantly different (95% CI 3.8 ± 1.06 µg.min/ml vs. 2.5 ± 0.212 µg.min/ml).

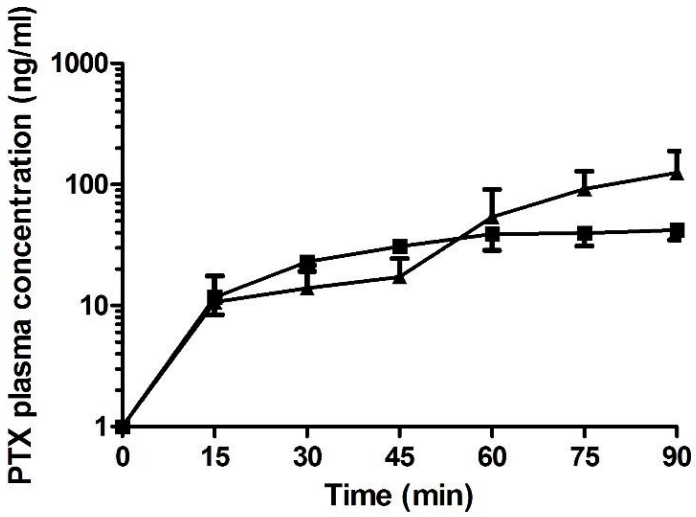

Figure 7. Paclitaxel plasma concentration (mean ± SD) (ng/ml) in rats (n=6) during and post-HIPEC treatment with a PTX concentration of 0.24 mg/ml delivered via (■) Taxol® and (▲) a PTX/ Plu F127 4/1 nanosuspension.

Previous research already described that nanoparticles (>50 nm) can adhere to mucosa, and thus prolonging the contact time of the drug and enhancing its absorption [10]. Prolonged retention of PTX in the peritoneal cavity (in combination with enhanced PTX penetration) can offer a therapeutic advantage as tumor cells are exposed to higher local drug levels for a longer time. This approach overcomes one of the limitations of conventional intraperitoneal (IP) drug therapy where drugs are rapidly cleared from the peritoneal cavity [12].

The effect of the PTX nanosuspension on tumor growth was evaluated via a tumor growth delay study, using Magnetic Resonance Imaging (MRI) as imaging technique to monitor tumor volume in a rat model. Although peritoneal carcinomatosis is characterized by a spread over the entire abdominal cavity, the rats were implanted with a single tumor nodule to simplify the tumor growth analysis (Fig. 8).

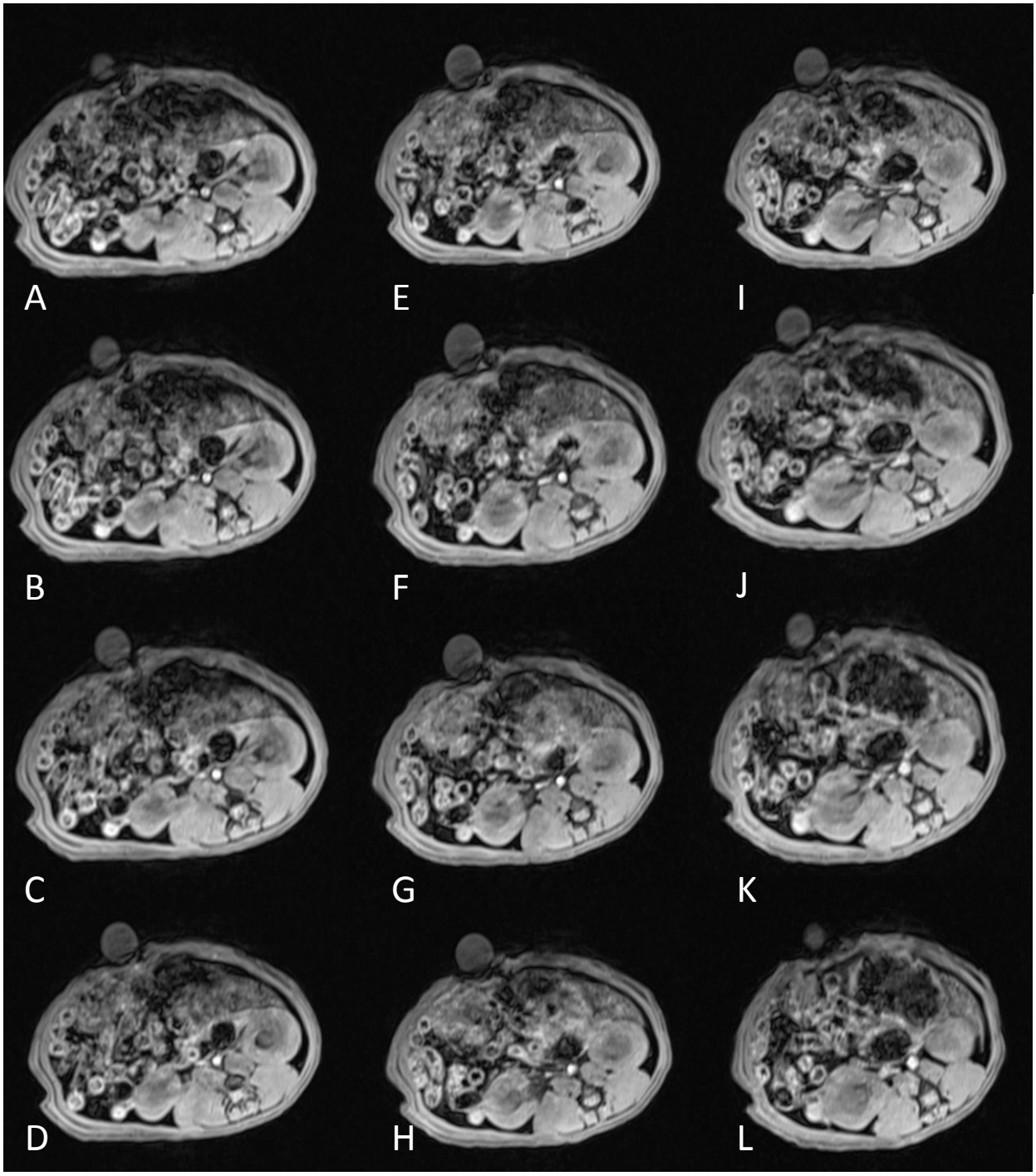

Figure 8. Transverse MRI images of a rat with a peritoneal tumor (situated under the vitamin B12 pellet). The volume of interest (VOI) consists of a stack of planar regions of interest (ROI's) (A->L).

At day 7 and 14 after HIPEC treatment with the PTX formulations, tumor growth was significantly different compared to the non-treated group (p=0.001 and 0.02 for Taxol®, and 0.003 and 0.010 for the PTX nanosuspension at day 7 and 14, respectively). No significant differences were observed between both PTX formulations (p=1.000 and p=0.929 at day 7 and

day 14, respectively) (Fig. 9). The effect of the PTX/Plu F127 nanosuspension on the tumor volume was similar to Taxol®. At day 7, tumor volume was reduced for both PTX formulations, although the results were not significantly different from the tumor volume at day 0 (p= 0.104 and 0.097 for Taxol® and the nanosuspension, respectively). At day 14, the tumor volume had increased compared to the volume at day 7 and was not significantly different from the initial tumor volume. Although little is known about the penetration of drugs in solid tumors, cytotoxic agents penetrate only a few millimeters into the tumor tissue, mostly via diffusion [21]. Due to the limited penetration of anticancer drugs in solid tumors, IP chemotherapy in the abdominal cavity is only effective in micrometastases or tumors smaller than 5 mm in diameter [22]. Hence, HIPEC could not completely eradicate the solid tumor implanted in the rat model and 14 days after treatment the tumor volume had increased as a result of proliferation of the remaining tumor cells. However, in clinical practice cytoreductive surgery precedes HIPEC treatment, while HIPEC is used to remove the remaining tumor cells which are not visible and also to prevent the implantation of tumor cells at the resection site and on other abdominal and pelvic surfaces [2]. Therefore, it is likely that in practice HIPEC using a PTX nanosuspension will remove all remaining microscopic tumors.

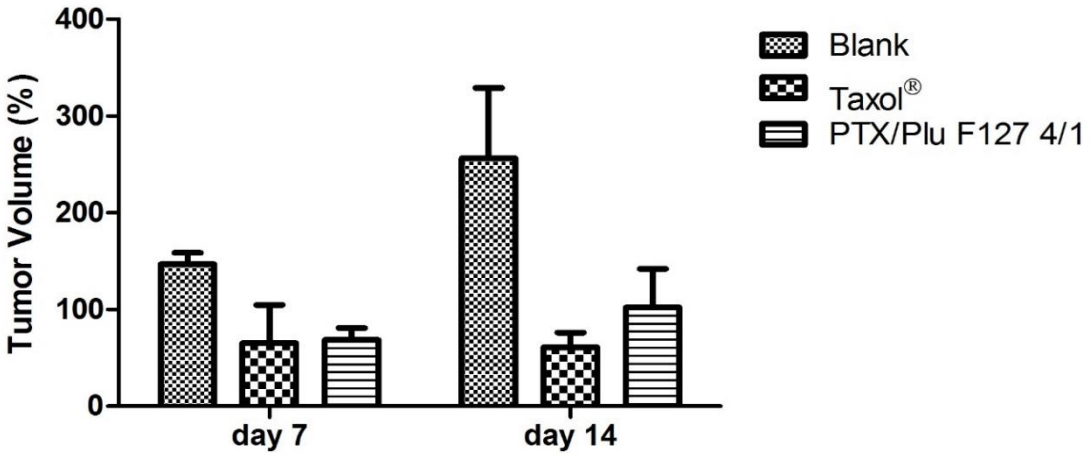

Figure 9. Tumor volume (mean ± SD) (%) compared to day 0, measured by MRI, 7 days and 14 days after HIPEC treatment with no treatment, Taxol® and PTX/Plu F127 4/1 nanosuspension (n=6).

CONCLUSION

A stable nanocrystalline paclitaxel formulation was developed via the wet milling technique using a high paclitaxel-to-stabilizer ratio. The cytotoxicity and antitumor efficacy in a rat model with peritoneal carcinomatosis of ovarian origin was similar to Taxol®. However, the advantage of using a non-cytotoxic excipient (Pluronic®F-127) in the nanosuspension was reflected by the faster recovery of the rat after HIPEC treatment.

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CHAPTER 2

HYPERTHERMIA FOR INTRAPERITONEAL CHEMOTHERAPY: IS THERE AN ADDITIONAL EFFECT IN COMBINATION WITH PACLITAXEL?

Manuscript of this chapter is in preparation:

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Hyperthermia for intraperitoneal chemotherapy: is there an additional effect in combination with paclitaxel?

INTRODUCTION

Ovarian cancer is the fifth most frequent cancer in females with approximately two thirds of the patients also presenting advanced disease, i.e. peritoneal carcinomatosis [1, 2], since the spread of cancer cells throughout the abdominal-pelvic cavity occurs very early in the development of the disease [3]. As ovarian cancer with tumor nodules on peritoneal surfaces can remain confined to the peritoneal cavity for a prolonged period of time without dissemination to other organs or regions, it makes peritoneal carcinomatosis of ovarian origin a good candidate for regional cancer treatment. Cytoreductive surgery followed by local intraperitoneal chemotherapy (IPEC) offers acceptable morbidity and mortality rates and increases the 5-year overall survival [4, 5]. The major goal of IPEC is to maximize the drug concentration in the abdominal cavity, in particular to target the tumor, while the systemic exposure is minimized [6]. Following IPEC a difference in drug concentration between the abdominal cavity and the systemic circulation occurs because drug transport from the peritoneal cavity to plasma (peritoneal clearance) is generally low, due to the presence of the peritoneal plasma barrier. In regional therapy the drug is delivered directly to the tumor, hence the therapeutic effect depends on the ability of the drug to penetrate in the tumor.

Hyperthermia, defined as an increase of temperature in the tumor-affected body region to 39 – 43 °C [7], is thought to increase drug penetration in solid tumors thereby increasing the cytostatic efficiency [8, 9]. Next to a direct cytotoxic effect, hyperthermia is believed to enhance the antitumor effect of several cytostatic agents by increasing tumor blood supply and oxygenation. It remains unclear if chemotherapy must be combined with hyperthermia to have a positive outcome on efficacy as it is known that hyperthermia causes serious side effects such as bone marrow suppression, renal failure, anastomic leakage and bowel perforation [10, 11].

PTX is a naturally occurring product extracted from the bark of the Taxus Brevifolia and is widely used for the treatment of breast, lung and advanced ovarian cancer [12]. The choice for PTX as drug for regional treatment of ovarian cancer is rational, because of its favorable pharmacokinetics as it has a high peritoneal/plasma concentration ratio (>1000) and a significant first pass effect [13]. Conflicting results have been reported from in vitro and in vivo

studies on the combination of PTX with hyperthermia [1]. The rationale for the use of hyperthermia with taxanes is based on the fact that mild hyperthermia results in a disorganization of the microtubule system, and taxanes are considered microtubule stabilizing agents. Thus, a common target of cytotoxic damage is involved.

However, PTX is not commonly used for IPEC treatment because of side effects (abdominal pain and life-threatening hypersensitivity reactions) reported for Taxol®, the initially available commercial formulation [14]. Therefore, novel PTX formulations have been developed without resorting to toxic excipients to improve the solubility of paclitaxel [15, 16]. Such as, Abraxane®, an albumin-bound PTX formulation which is already available on the market for the treatment of metastatic breast cancer [17] and Genexol®PM, a polymeric micellar PTX formulation, which shows good results for the treatment of breast, lung and pancreatic cancer [18]. However, both formulations were not yet evaluated for the treatment of advanced ovarian cancer.

In this study, the effect of hyperthermia in combination with 3 different paclitaxel formulations, Abraxane®, Genexol®PM and Taxol®, was evaluated for (H)IPEC for the treatment of peritoneal carcinomatosis of ovarian origin. The toxicity (maximum tolerated dose, recovery time and bioavailability) and efficacy (tumor growth delay and PTX tumor accumulation) of these treatments were evaluated following (H)IPEC.

MATERIALS AND METHODS

Materials

Three commercially available paclitaxel (PTX) formulations were used: Taxol® (1:1 Cremophor®EL/ethanol mixture) from Bristol-Myers Squibb (Brussels, Belgium), Genexol®PM (polymeric-micellar formulation using a biodegradable monomethoxy poly(ethylene glycol)-block-poly(D,L lactide) block copolymer) from Samyang (Seoul, Korea) and Abraxane® (PTX albumin-bound particles) from Abraxis Bioscience (London, UK).

In vitro cytotoxicity

An ovarian carcinoma cell line (SKOV-3, obtained from the American Type Culture Collection) was cultured at 37 °C in a 5% CO_2-containing humidified atmosphere. SKOV-3 cells were cultured in McCoys medium (Invitrogen, Merelbeke, Belgium). The medium was supplemented with 10% fetal bovine serum, penicillin, streptomycin (Invitrogen, Merelbeke, Belgium) and fungizone (Bristol Myers Squibb, Brussels, Belgium).

The cytotoxicity of the different PTX formulations at different temperatures (37 °C and 41.5 °C) was tested at PTX concentrations of 0.01, 0.1, 1, 5 and 10 µg/ml, 8 wells per concentration were used.

To evaluate the cytotoxicity, $20x10^3$ cells/ml were seeded in 96-well plates (Sarstedt, Newton NC, USA). After 72 hours, 20 µl medium was removed and replaced by the test formulation. After incubation for 1 hour both at 37 °C or 41.5 °C (i.e. to mimic the HIPEC procedure used during in vivo studies), the medium was entirely removed and replaced by 200 µl fresh medium in each well. Afterwards, the cells were incubated for 24 and 96 hours at 37 °C under 5% CO_2-atmosphere. The cytotoxicity of the test formulations was determined via MTT assay and compared with the non-treated cells. The MTT procedure is described in Chapter 1. Afterwards, the optical density was measured at 570 nm normalizing with a reference wavelength of 650 nm using an ELISA-plate reader (Paradigm Detection Platform, Beckman Coulter, Suarleé, Belgium).

(H)IPEC procedure

Female athymic nude rats (Harlan, Horst, The Netherlands) were kept in standard housing conditions with water and food ad libitum and a 12 hours light/dark circle. All animal experiments were approved by the institutional review board of the Faculty of Medicine, Ghent University (ECD 09/06).

After anesthetizing each rat with 3% isoflurane (Forene®, Abbott, Waver, Belgium) a vertical incision was made along the midline in the abdominal wall muscle. The abdominal wall muscle was attached to a metal ring which was placed a few centimeters above the incision. The inlet

and outlet tubing (Pumpsil®, Watson-Marlow, Zwijnaarde, Belgium) was placed in the peritoneal cavity for perfusion with the cytostatic solution over a period of 45 min. To evaluate the influence of the temperature the solution was passed through a heat exchanger set at 37 °C (IPEC) or 41.5 °C (HIPEC). During perfusion, the perfusate solution and body temperature of the rat were closely monitored and data were collected using E-Val® 2.10 Software (ELLAB®, Roedovre, Denmark).

Maximum tolerated dose

The maximum tolerated dose (MTD) was determined for all PTX formulations at HIPEC settings (i.e. 45 min perfusion at 41.5 °C). MTD was defined and determined as described by Bouquet et al. [19]. Based on the MTD of Taxol® (0.24 mg/ml) [20], a PTX concentration of 0.21 mg/ml was used as starting point. In combination with the MTD, the time to regain their initial body weight (recovery time) was also evaluated. The recovery time was evaluated for rats treated under normothermic and hyperthermic conditions. The time to regain the initial body weight was evaluated with a 2-way ANOVA with a significance level of 0.05 (SPSS 19.0).

Bioavailability

Blood was sampled in heparin-containing tubes via a catheter which was placed in the arteria carotis. Blood samples were taken at 0, 15, 30, 45, 60 and 90 min after starting the perfusion. Perfusate samples were collected at 0, 15, 30 and 45 min after starting perfusion, to ensure that an accurate dose was administered during (H)IPEC procedure. After the bioavailability study rats were euthanized. Blood samples were immediately centrifuged, and separated plasma was stored at -80 °C until analysis.

The perfusate and plasma samples were analysed as previously described in Chapter 1.

For the bioavailability study, the pharmacokinetic parameters of the PTX treatment groups were compared using a 2-way ANOVA with a significance level of 0.05 (SPSS 19.0). The AUC was log-transformed before analysis.

Tumor model

Donor rats were injected with $30x10^6$ SKOV-3 cells between the peritoneum and the abdominal muscle to induce a tumor. The animals received a daily subcutaneous injection of 3 mg cyclosporine over a period from 3 days prior until 10 days after tumor cell injection. After 3 to 4 weeks, tumor size was sufficiently enlarged to transplant tissue samples (5 x 5 mm, with a thickness of 3 mm) on the parietal peritoneum of an acceptor rat. The acceptor rat also received daily subcutaneous injection of 3 mg cyclosporine from 3 days prior until 10 days after tumor transplantation to ensure tumor attachment. Two weeks after transplantation, the tumor had attached to the peritoneum and was sufficiently grown to perform the tumor growth delay (TGD) experiment and determine PTX accumulation in the tumor.

Tumor growth delay study

The effect of the PTX formulations on tumor growth was evaluated via a Siemens® Trio 3T clinical MRI scanner (Erlangen, Germany). Prior to the MRI scan, rats were anaesthetized with Rompun 2% (Bayer, Diegem, Belgium) and ketamine 1000 CEVA (Ceva, Amersham, UK) using a dose of 10 mg/kg and 90 mg/kg, respectively. The rats were placed prone in a (wrist) coil to measure the tumor volume. A T1-weighted 3D FLASH sequence was applied with a flip angle of 10°, a repetition time of 13 ms and an echo time of 4.9 ms to obtain a voxel size of 0.19 x 0.19 x 0.4 mm^3. In order to easily locate the tumor, the rat was palpated and a vitamin B12 pellet was attached to the skin where the tumor was located. Tumor volume was defined by creating a volume of interest (VOI) consisting of a stack of planar regions of interest (ROI's) using PMOD software (PMOD Technologies, Adliswil, Switserland). Rats were scanned 1 day before (H)IPEC treatment (day 0) to measure the initial volume of the tumor. At day 1 rats were treated with PTX formulations. Tumor volume was evaluated 7 and 14 days after (H)IPEC treatment to monitor the effect of the formulations.

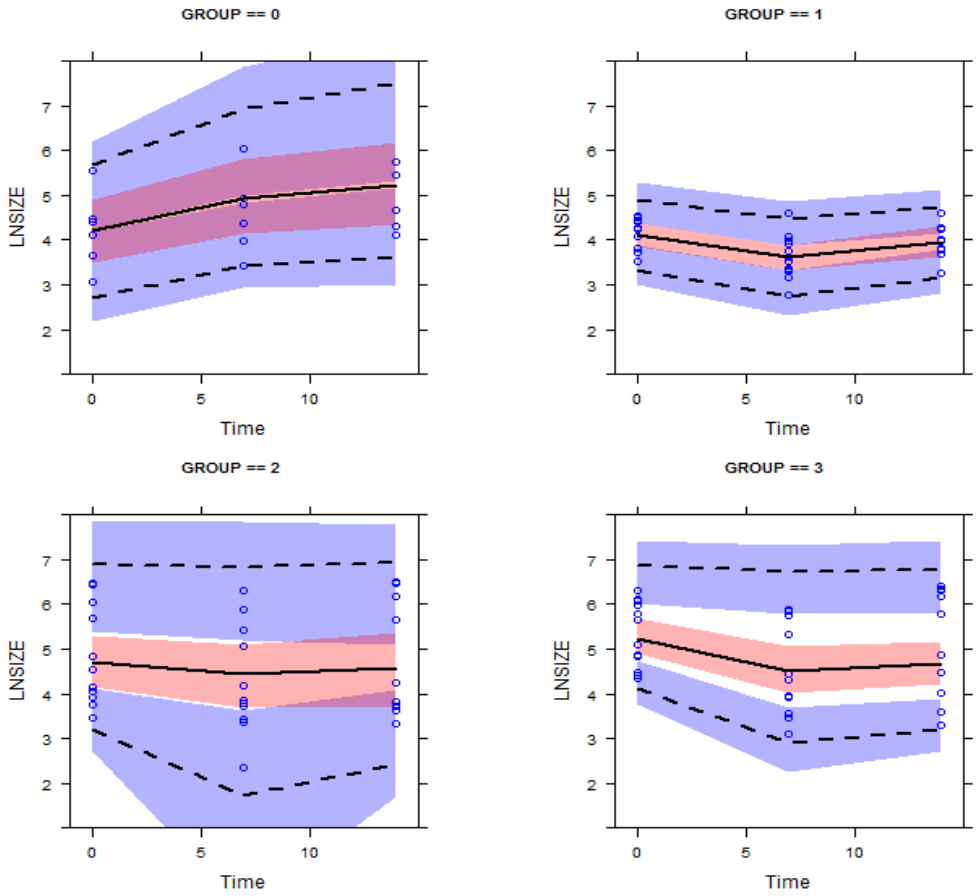

Figure 1. Visual predictive check plots for the different paclitaxel formulations. Groups 0, 1, 2 and 3 correspond to the non-treated group, Taxol®, Genexol®PM and Abraxane® respectively. Blue circles denote observed ln-transformed tumor sizes at different measurement times. Solid black lines indicate model predicted median ln-transformed tumor sizes whereas dashed black lines represent model predicted 2.5 and 97.5 percentiles. The blue and red areas represent the confidence regions around the model predicted 2.5 and 97.5 percentiles and the median respectively.

For the TGD study, 120 tumor sizes were measured, originating from 42 rats at 3 different measurement occasions (day 0, day 7 and day 14), divided in 7 groups (the non-treated group and Taxol®, Abraxane®, Genexol®PM, each consisting of a normo- and hyperthermia group). The tumor sizes were ln-transformed prior to analysis using a linear mixed effects model. Parameter estimates were obtained using LAPLACE estimation method in NONMEM® (Version 7.2, GloboMax LLC, Hanover, US).

For the blank group the linear rate of tumor growth as well as the inter-individual variability in tumor growth was estimated. For the different formulations, the baseline measurements

as well as the inter-individual variability in the formulation-specific baseline measurements were modeled. To accommodate the apparent biphasic effect of the different formulation on tumor growth, a piecewise-linear regression model was used. For each formulation a parameter was estimated describing the decrease in tumor volume between day 0 and day 7 and another parameter was estimated to describe the tumor regrowth between day 7 and day 14.

To elucidate the potential interaction between hyperthermia and the PTX formulations, additional parameters estimating the difference between formulation effects of the normothermic and hyperthermic groups were added to the model. A visual predictive check provides evidence of adequate model fit for the data (Fig. 1).

Paclitaxel tumor penetration

After rinsing and weighing, samples were suspended in water and tissue was mechanically disrupted. Subsequently, internal standard (C^{13}-paclitaxel) was added. Tumor tissue was then enzymatically digested using a proteinase-K and a lipase solution (both Sigma-Aldrich, Diegem, Belgium). Tissue suspensions were left to incubate overnight at 55 °C whilst continuously being shaken (IKA®, Staufen, Germany).

After centrifugation of the digested tissue suspension at 10.000xg for 20 min, supernatant was collected and subjected to solid phase extraction. In short, after loading of the sample, the SPE cartridges (Oasis® HLC cartridges, Waters, USA) were washed with a sodium hydroxide (pH 10.0) solution and a 70% (v/v) methanol solution in water. Following elution of PTX, samples were concentrated by evaporation of methanol under a mild nitrogen stream (30 min, 35 °C). Finally, after reconstitution, samples were injected onto the Waters Acquity UPLC system (BEH C18 column). Paclitaxel and C^{13}-paclitaxel were detected with a Quattro Ultima triple quadrupole system (Micromass Waters, Manchester, UK). The measurement range of the analytical method was 2.0 – 500 ng/g. Total imprecision and trueness were calculated on results of repeated analysis of quality controls and complied with the FDA guidance specifications on maximum tolerable bias and imprecision for all QC levels. (Trueness: 93.3 – 98.0 – 96.5; Imprecision: 20.4 – 3.2 – 1.8; both expressed as %).

RESULTS

In vitro cytotoxicity

The cytotoxicity of the PTX formulations in combination with or without hyperthermia was evaluated on SKOV-3, an ovarian carcinoma cell line. Figure 2 shows no significant differences between the formulations at different time points. Although the PTX incubation time was short (PTX was removed after 1 hour), the cytotoxic effect (for the highest PTX dose) increased in function of time: 81 ± 1%, 71 ± 9% and 68 ± 1% after 24 hours vs. 17 ± 2%, 19 ± 1% and 19 ± 1% after 96 hours for Abraxane®, Taxol® and Genexol®PM respectively, under normothermic conditions. Lower PTX concentrations (0.01 – 0.1 µg/ml) gave no reduction of cell viability under normothermic conditions, whereas cells treated under hyperthermic conditions showed a reduction of cell viability after 96 hours : 76 ± 2%, 72 ± 8% and 64 ± 4% for Abraxane®, Taxol® and Genexol®PM, respectively. Adding hyperthermia to higher PTX concentrations (1 – 10 µg/ml) showed no additional decrease of cell viability.

Table I. Toxicity of different paclitaxel formulations after (H)IPEC treatment at normothermic and hyperthermic conditions.

Treatment		Days to regain 100% body weight
Taxol®	41.5 °C	13.4 ± 1.7
	37 °C	10.2 ± 0.6
Genexol®PM	41.5 °C	6.4 ± 0.6
	37 °C	3.8 ± 0.9
Abraxane®	41.5 °C	7.8 ± 0.8
	37 °C	6.1 ± 0.5

Evaluation of the toxicity

The maximum tolerated dose (MTD) of the PTX formulations was determined by monitoring the survival rate and body weight of the rats following HIPEC treatment. For Abraxane® and Taxol® a PTX concentration of 0.27 mg/ml resulted in mortality, while the PTX concentration

could be increased to 0.3 mg/ml before mortality was observed in rats treated with Genexol®PM. Hence, 0.24 mg/ml was set as MTD for Abraxane® and Taxol®, and 0.27 mg/ml for Genexol®PM. At a PTX dose of 0.24 mg/ml, rats treated with Abraxane® and Genexol®PM recovered in approximately one week, while rats treated with Taxol® needed 13.4 ± 1.7 days to regain their initial body weight (Table I). These results suggested a lower toxicity for Genexol®PM and Abraxane® compared to Taxol® ($p=0.00$). Moreover, for all formulations, rats treated under normothermic conditions recovered significantly faster compared to those treated at hyperthermic conditions ($p<0.05$).

Since the toxicity of a formulation intended for local intraperitoneal drug administration is related to the systemic uptake, the rationale for (H)IPEC is to maximize local PTX concentration, thus enhancing drug penetration in tumor tissue while minimizing systemic uptake. Maximum plasma concentrations were detected 30 min after starting (H)IPEC for Abraxane® and Genexol®PM, and after 60 min for Taxol® both under normothermic and hyperthermic conditions. Monitoring of PTX plasma concentrations was limited to a 60 min period, since previous research [20] showed no further increase in PTX plasma concentrations after removing the cytotoxic solution from the peritoneal cavity (i.e. after 45 min).

Table II. Geometric pharmacokinetic parameters (± SD) of different paclitaxel formulations after (H)IPEC treatment.

Treatment		$AUC_{IP,\ t=45min}$ (mg.min/ml)	$AUC_{PI,\ t=60min}$ (µg.min/ml)	$AUC_{t=45\ min}$ ratio*
Taxol®	37 °C	7.0 ± 1.4	2.1 ± 1.1	4995 ± 1539
	41.5 °C	6.5 ± 1.1	2.6 ± 0.8	4940 ± 1945
Genexol®PM	37 °C	6.2 ± 0.8	3.5 ± 1.2	3451 ± 805
	41.5 °C	6.5 ± 1.0	4.0 ± 1.6	3355 ± 1099
Abraxane®	37 °C	7.2 ± 1.2	11.3 ± 1.5	609 ± 465
	41.5 °C	7.6 ± 1.6	14.7 ± 1.9	829 ± 523

*area under the curve ratio of intraperitoneal PTX concentration and plasma PTX concentration over a period of 45 min

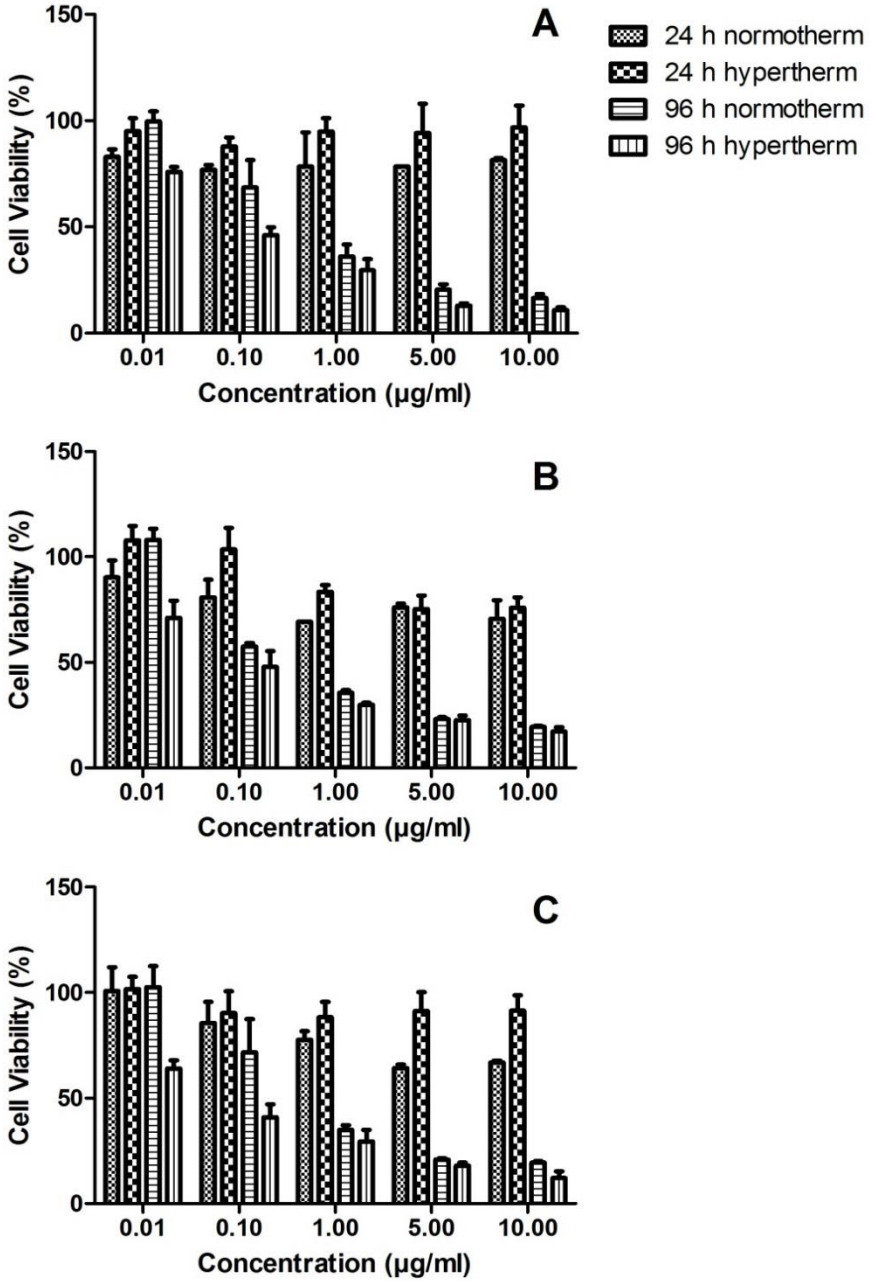

Figure 2. Viability of the SKOV-3 cell line after application of different paclitaxel formulations at normothermic (37 °C) and hyperthermic (41.5 °C) conditions. (A) Abraxane®,(B) Taxol® and (C) Genexol®PM.

Intraperitoneal perfusion with Taxol® and Genexol®PM resulted in similar, non-significant pharmacokinetic profiles, whereas perfusion with Abraxane® yielded higher PTX plasma concentrations compared to Taxol® and Genexol®PM (Fig. 3). The different IP clearance of PTX was also reflected in the pharmacokinetic parameters (Table II). Two-way ANOVA showed no

significant differences of $AUC_{PI,\ t=60min}$ and C_{max} between normo- and hyperthermic conditions (p=0.09). In contrast the $AUC_{PI,\ t=60min}$ of Abraxane® was significantly higher both under normothermic and hyperthermic conditions compared to the Taxol® and Genexol®PM groups (p=0.00). During (H)IPEC the PTX concentrations in the abdominal cavity were much higher than in plasma (Table II) for all PTX formulations, resulting in high AUC ratio's. The AUC ratio of Abraxane® is significantly lower (p<0.05) compared to the other two formulations. As no significant differences in AUC_{IP} were observed between the formulations and the administered concentration was similar, a higher systemic toxicity would be expected after (H)IPEC treatment with Abraxane®.

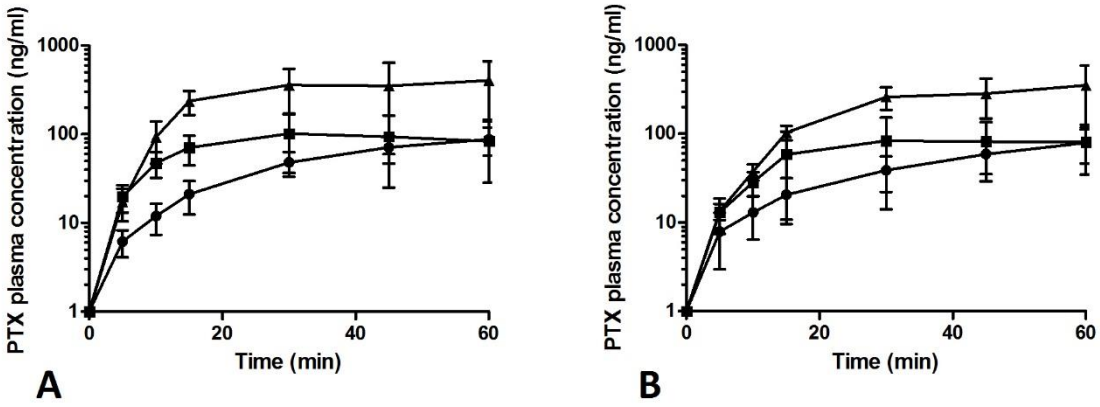

Figure 3. Paclitaxel plasma concentration (ng/ml) (± SD) in rats (n=6) after HIPEC treatment with a paclitaxel concentration of 0.24 mg/ml delivered via (▲) Abraxane®, (■) Genexol®PM and (●) Taxol® under (A) hyperthermic conditions and (B) normothermic conditions.

Evaluation of the efficacy

No differences in PTX tumor accumulation were observed between the normothermic and hyperthermic groups (p>0.05) (Table III). Also no differences (p>0.05) in PTX concentrations were observed between the different PTX treatment groups. In comparison with the plasma concentration high PTX concentrations were found in the tumor.

Table III. Paclitaxel tumor accumulation (± SD) after (H)IPEC treatment (n=4).

Treatment		PTX tumor concentration (µg/g)
Taxol®	37 °C	10.5 ± 5.1
	41.5 °C	8.4 ± 1.7
Genexol®PM	37 °C	9.8 ± 3.4
	41.5 °C	10.9 ± 5.5
Abraxane®	37 °C	8.3 ± 1.9
	41.5 °C	7.2 ± 1.4

Based on the linear regression model (equation 1), an estimation of the mean tumor growth in function of time was made (Fig. 4). For the non-treated group (solid line) tumor volume increased in function of the time, while the PTX treated rats showed a significant decrease in tumor volume till 7 days after the treatment. As the rats were implanted with a single tumor nodule (>0.5 cm) to simplify analysis, the tumor was not completely eradicated by (H)IPEC and tumor volume increased again between day 7 and day 14 for all PTX formulations.

Equation 1. Piecewise-linear regression model to predict tumor volume including the hyperthermic effect.

$$TIME < 7\ days: Y_{i,j,k} = Int_{j,k} + TIME_i * \beta_{1,j,k} * I_{1,j,k}$$

$$TIME \geq 7\ days: Y_{i,j,k} = Int_{j,k} + TIME_i * \beta_{1,j,k} * I_{1,j,k} + (TIME_i - 7) * \beta_{2,j,k} * I_{2,j,k}$$

With $I_{n,j,k} = \begin{cases} 1, & IF\ group = Normotherm \\ \beta_{n,j,k}, & IF\ group = Hypertherm \end{cases}$

With $\varepsilon_i \sim e^{N(0,\sigma^2)}$

With:

I = hyperthermic interaction term, Int = Intercept (mm^3), β = Slope (mm^3/day)

i (time) = 0, 7, 14 days

j (treatment groups) = Placebo, Taxol®, Genexol®PM, Abraxane®

k (temperature) = 0 (normotherm), 1(hypertherm)

Despite the increase of tumor volume at day 14, all formulations had a significant decrease in tumor growth compared to the non-treated group which had a tumor volume of 147 ± 12% and 257 ± 72% at day 7 and day 14, respectively (Fig. 5). Comparing the different PTX formulations showed a significant decrease in tumor volume for the Abraxane®-treated group (p=0.0073) between day 0 and 7 compared to the Taxol® and Genexol®PM-treated rats which showed a similar evolution of tumor growth (Table IV). To elucidate the potential interaction effect of hyperthermia with the PTX formulations, additional parameters estimating the relative difference between the formulation effects in the normothermic versus hyperthermic groups were added to the model. No significant decrease of tumor volume was observed by adding hyperthermia to the therapy as the estimated interaction term was not significantly different from 1 (Table IV, gray shaded area).

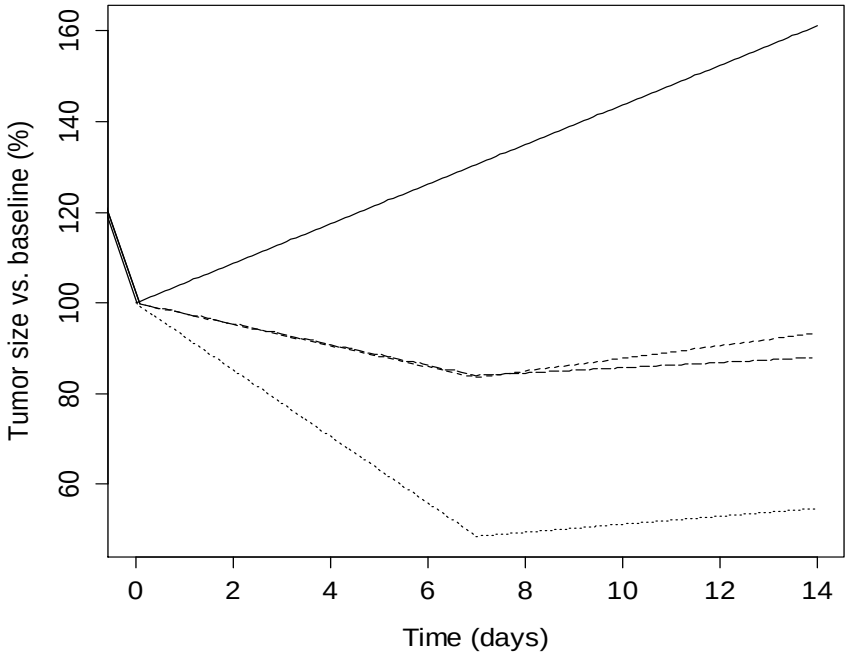

Figure 4. Model estimated tumor size versus baseline for the different paclitaxel formulations. The black solid line represents the placebo group whereas the dashed lines represent model predictions for the Taxol® and Genexol®PM-treated rats. The model predictions for the Abraxane® group are represented by the black dotted line.

Table IV. Parameter estimates of the effects on tumor growth and the additional effect of hyperthermia.

INTERCEPT (Int) (mm^3)				
Parameter	Estimate	SE	RSE (%)	95% CI
Blank	66.9	19.4	29.0	28.88 – 104.92
NORM Taxol®	62.1	6.15	9.9	49.95 – 74.05
NORM Genexol®PM	75.2	21.6	28.7	32.86 – 117.54
NORM Abraxane®	363.0	46.5	12.8	271.86 – 454.14
HYP Genexol®PM	122.0	22.6	18.5	77.70 – 166.30
HYP Abraxane®	183.0	80.3	43.9	25.61 – 340.39
SLOPE (β) (mm^3/day)				
Parameter	Estimate	SE	RSE (%)	95% CI
Blank (day 0 –> day 14)	6.5	3.0	45.9	-0.65 – 12.37
Taxol® (day 0 –> day 7)	-3.6	0.65	17.9	-4.86 – -2.34
Genexol®PM (day 0 –> day 7)	-3.2	0.95	29.6	-5.09 – -1.35
Abraxane® (day 0 –> day 7)	-9.5	3.6	37.4	-16.46 – -2.54
Taxol® (day 7 –> day 14)	4.4	1.1	24.9	2.25 – 6.57
Genexol®PM (day 7 –> day 14)	4.4	1.2	27.6	2.04 – 6.86
Abraxane® (day 7 –> day 14)	10.3	2.9	27.8	4.69 – 15.90
HYPERTHERMIC INTERACTION TERM (I)				
Parameter	Estimate	SE	RSE (%)	95% CI
HYP Taxol® (day 0 –> day 7)	0.95	0.31	32.7	0.34 – 1.56
HYP Taxol® (day 7 –> day 14)	1.6	0.75	47.7	0.10 – 3.04
HYP Genexol®PM (day 0 –> day 7)	1.2	0.69	56.6	-0.13 – 2.55
HYP Genexol®PM (day 7 –> day 14)	0.98	0.73	74.9	-0.46 – 2.41
HYP Abraxane® (day 0 –> day 7)	1.2	0.30	25.7	0.58 – 1.74
HYP Abraxane® (day 7 –> day 14)	1.2	0.38	31.6	0.46 – 1.96

DISCUSSION

In many institutions IPEC is performed under hyperthermic conditions as hyperthermia not only acts in a cytotoxic way itself but also sensitizes tumor cells to radiotherapy (thermal radiosensitization) and various cytostatic drugs (thermal chemosensitization). Hyperthermia should also increase the penetration of cytostatic drugs into the tumor.

Conflicting results have been reported from in vitro and in vivo studies on the combination of PTX with hyperthermia [1]. The rationale for the use of hyperthermia with taxanes is based on the fact that both interact with the microtubule system. In this study 3 different PTX formulations were used in order to exclude the toxic effect of Cremophor®EL present in the commercially available formulation Taxol®. Weiszhár et al. [21] identified that the side effects following administration of Taxol® are mainly due to the presence of Cremophor®EL.

Based on the results of the MTT test, no differences in cytotoxic effect were observed between the different PTX formulations. On the other hand, hyperthermia has a cytotoxic effect at low PTX concentrations but this effect was undone by the strong cytotoxic effect of PTX at higher concentrations. Since hyperthermia has no additional or synergetic effect in vitro combined with PTX, the hypothesis that hyperthermia would increase the drug entry into the cells [22] is not valid for PTX on SKOV-3 cells.

As (H)IPEC is a promising therapy for the treatment of peritoneal carcinomatosis of ovarian origin and PTX is an ideal molecule for (H)IPEC treatment due to its pharmacokinetic profile and significant first pass effect, it is important to optimize the PTX treatment protocol. (H)IPEC treatment is characterized by high local doses resulting in a high efficacy and a low toxicity. In this study, the toxicity was examined by the maximum tolerated dose, the recovery time of the rats and the bioavailability of PTX in the plasma, as these concentrations are a prediction for the PTX induced side effects such as neutropenia and neuropathy.

Based on the results of the toxicity studies, Genexol®PM presents a suitable alternative to Taxol®, as it has a similar pharmacokinetic profile but a lower toxicity (higher MTD and faster recovery compared to Taxol®) considering the absence of the toxic surfactant, Cremophor®EL. In contrast to systemic therapy where drug delivery to the tumor cells occurs via the

circulation, drug delivery to tumor cells during local therapy depends in the first place on the ability of the drug to penetrate the tumor by diffusion through tumor interstitium. The recirculation of the drug absorbed from the peritoneal cavity is of lower importance.

The efficacy of the treatment was analysed by the penetration of PTX in the tumor and by a TGD study. While some studies [23, 24] show that hyperthermia increases drug accumulation in the tumor, our results showed no significant differences in PTX tumor accumulation compared to the normothermic treatment group. Although no differences in PTX tumor penetration between the different formulations were observed, Abraxane® showed a higher decrease in tumor volume compared to Genexol®PM and Taxol®. This can suggest that the increased effect of Abraxane® which also has higher PTX plasma levels, is determined by the PTX tumor penetration but also by the recirculation of PTX. As already described, (H)IPEC treatment is characterized by high local doses resulting in a high efficacy and a low toxicity. Both Abraxane® and Genexol®PM, in a different way, meet these requirements.

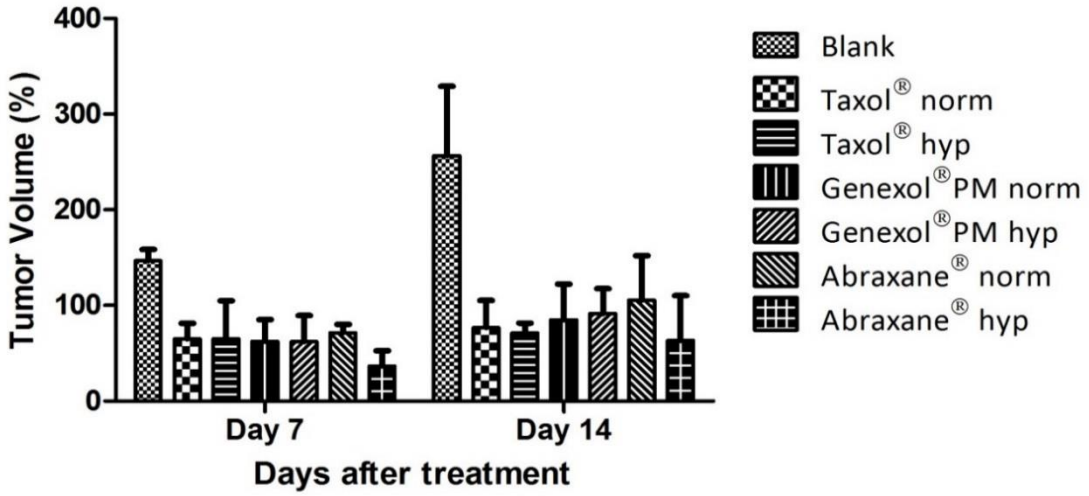

Figure 5. Tumor volume (mean ± SD) (%) compared to day 0, measured by MRI 7 days and 14 days after (H)IPEC treatment (n=6).

One has to wonder whether the slightly higher effect on tumor growth caused by Abraxane® compensates its higher systemic toxicity. In spite of the differences between the different treatment groups, all tumor nodules showed a significant volume reduction compared to the

non-treated animals. But after treatment, the remaining tumor cells restarted to proliferate and after 7 days the tumor size increased again. Due to the limited penetration of anticancer drugs, cytostatic drugs only penetrate a few millimeters into the tumor tissue, IP chemotherapy is only effective in micrometastases or tumors smaller than 5 mm in diameter [25]. However, in clinical practice cytoreductive surgery removes first all visible tumor nodules prior to HIPEC treatment which is used to remove all microscopic disease (<0.5 cm) and to prevent implantation of tumor cells at the resection site and on other peritoneal surfaces. Therefore, one can assume that in practice all remaining microscopic tumors will be removed by (H)IPEC treatment.

Although the direct cytotoxic effect of heat is already known, conflicting results have been reported regarding the interaction of heat and taxanes. Debree et al. reviewed studies regarding thermal enhancement on PTX therapy [1], where all studies varied in drug concentration, exposure time, degree and duration of hyperthermia and cell type. Only two studies [26, 27] showed an increased cytotoxic effect when PTX was combined with hyperthermia. In our study no enhancement was seen under hyperthermic conditions when cancer cells were exposed to a high local PTX concentration. These data indicated that hyperthermia is less preferred in intraperitoneal perfusion therapy as it results in a similar bioavailability and tumor reduction, but a longer recovery time compared with normothermic perfusion.

CONCLUSION

Hyperthermia in combination with intraperitoneal perfusion did not present an added value as it resulted in a similar efficacy and a higher toxicity (a longer recovery time) compared with the normothermic treatment group. Both Abraxane® and Genexol®PM are suitable formulations for IPEC treatment of peritoneal carcinomatosis of ovarian origin. Genexol®PM meets all requirements for IPEC treatment: administration of high doses with a low systemic exposure and a limited toxicity but the TGD study showed a lower decrease in tumor volume compared to Abraxane®, which has a higher systemic absorption. To draw any conclusion about the best alternative formulation for Taxol® further evaluation of the systemic toxicity of Abraxane® is needed.

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CHAPTER 3

PACLITAXEL TUMOR PENETRATION AFTER INTRAPERITONEAL CHEMOTHERAPY

Manuscript of this chapter is in preparation:

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INTRODUCTION

Little is known about the penetration and distribution of cytotoxic agents within solid tumors. After intravenous therapy, the cytotoxic agent must be delivered to the tumor by its imperfect blood vasculature, cross vessel walls into the interstitium and penetrate multiple layers of solid tissue [1]. When the cytotoxic drug is in direct contact with the tumor, as in regional therapy, drug delivery to the tumor cells occurs primarily by diffusion through the interstitial space [2]. Intraoperative intraperitoneal chemotherapy (IPEC) is an example of regional therapy and is used for the treatment of malignancies which are confined to the abdominal cavity such as ovarian, colon and appendiceal cancer [3, 4]. The strong rationale behind this therapy has translated into an increased 5-year survival for patients with ovarian cancer [5].

The principal idea of IPEC is to expose cancer cells within the abdominal cavity to high local drug concentrations, while minimizing the systemic side effects [6]. Although the results for IPEC treatment are very promising, there are still many uncertainties about the treatment protocol. No standard treatment in terms of schedule, residence time, drug, or carrier solution has been established. One of the limiting factors for IPEC is the limited penetration of the cytostatic drugs into the tumor [7]. Several studies, for different cytotoxic agents, were already performed to determine the penetration profile after intraperitoneal (IP) treatment. In spite of the high local concentrations, the direct penetration of these drugs into the tumor is limited, ranging from several cell layers to 1 – 3 mm [8]. There is strong evidence that the antitumor activity of cytostatic drugs is closely related to the amount of drug reaching the tumor, so the limited penetration may limit the antitumor effect, but it may also protect the healthy cells on the mucosal side of the gastrointestinal tract. Successful chemotherapy induces cell death, including apoptosis, or programmed cell death, which indicates tumor response to the therapy [9, 10].

In this study, the tumor penetration of paclitaxel (PTX), a highly protein-bound drug, widely used for the treatment of breast, lung, and advanced ovarian cancer [11], was evaluated after IPEC treatment. PTX has a low water solubility, a high molecular weight and undergoes a significant first pass metabolism in the liver [12] which makes it a promising molecule for IP treatment because of limited absorption from the peritoneal cavity. The unbound

concentration of PTX penetrates into the tumor cells and shows a high affinity with the intracellular binding sites. These intracellular concentrations are critical for the cytotoxic effect of PTX. The efficacy of the penetration was evaluated by scoring the apoptosis. In a second phase, an exploratory study was performed in order to characterize the tumor penetration, systemic pharmacokinetics (PK) and pharmacodynamics (PD) of PTX after IPEC (by varying the contact time and dose of the treatment).

MATERIALS AND METHODS

Materials

Taxol® (6 mg/ml PTX in a 1:1 Cremophor®EL/ethanol mixture) from Bristol-Myers Squibb (Brussels, Belgium) was used as model PTX formulation in order to evaluate the penetration of PTX.

Tumor model

A human ovarian carcinoma cell line (SKOV-3, obtained from the American Type Culture Collection) was cultured at 37 °C in a 5% CO_2-containing humidified atmosphere. SKOV-3 cells were cultured in McCoys medium (Invitrogen, Merelbeke, Belgium) which was supplemented with 10% fetal bovine serum, penicillin, streptomycin (Invitrogen, Merelbeke, Belgium) and fungizone (Bristol Myers Squibb, Brussels, Belgium).

Adult female athymic nude rats (Harlan, Horst, The Netherlands) were kept in standard housing conditions with water and food ad libitum and a 12 hours light/dark circle. All animal experiments were approved by the Ethical Committee of the Faculty of Medicine, Ghent University (ECD 09/06).

Donor rats were injected with $30x10^6$ SKOV-3 cells in a volume of 0.2 ml between the peritoneum and the abdominal muscle to induce a tumor. The animals received a daily subcutaneous injection of 3 mg cyclosporine over a period from 3 days prior until 10 days after tumor cell injection. After 3 to 4 weeks, tumor size was sufficiently enlarged to transplant tissue samples (5 x 5 mm, with a thickness of 3 mm) on the parietal peritoneum of an acceptor

rat. The acceptor rat also received daily subcutaneous injections of 3 mg cyclosporine from 3 days prior until 10 days after tumor transplantation to ensure tumor attachment. 3 weeks after transplantation, the tumor was attached to the peritoneum and had sufficiently grown to perform the IPEC.

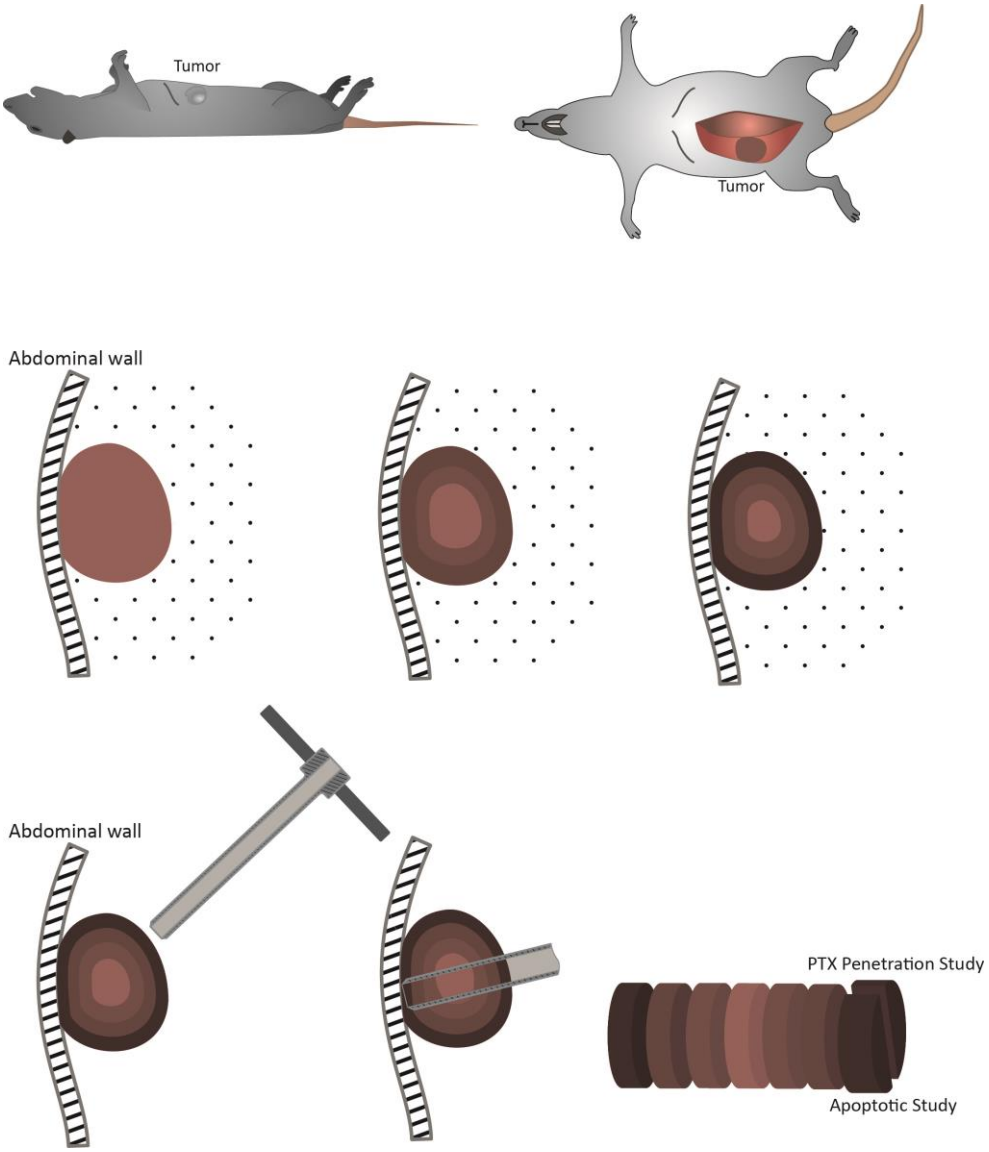

Figure 1. Paclitaxel penetration in the tumor after IPEC treatment and harvesting the tumor for the paclitaxel penetration study and the apoptotic study.

IPEC procedure

After anesthetizing each rat with 3% isoflurane (Forene®, Abbott, Waver, Belgium) a vertical incision was made along the midline in the abdominal wall muscle. The abdominal wall muscle

was attached to a metal ring which was placed a few centimeters above the incision. The inlet and outlet tubing (Pumpsil®, Watson-Marlow, Zwijnaarde, Belgium) was placed in the peritoneal cavity for perfusion with the cytostatic Taxol® solution, a PTX dose of 3 mg or 30 mg was used in a volume of 125 ml 0.9% NaCl. The highest dose was previously determined as the maximum tolerated dose in female athymic nude rats [13]. A roller pump (Watson-Marlow, Zwijnaarde, Belgium) circulated the cytostatic solution through a heat exchanger set at 37 °C. During perfusion, the perfusate solution and body temperature of the rat were closely monitored and data was collected using E-Val® 2.10 Software (ELLAB®, Roedovre, Denmark). IPEC procedure was never performed longer than 45 min and afterwards the abdominal cavity was flushed with 0.9% NaCl solution and sutured.

Tumor harvesting

To evaluate the PTX tissue concentration and the apoptosis in function of time, the tumor was harvested at different time points (5, 15, 30, 45, 60, 90, 120, 240 and 480 min) after starting IPEC treatment. After harvesting the tumor, rats were euthanized. A biopsy of the tumor was taken in order to obtain cylinder-shaped tumor tissue (± 10 mm in diameter) (Fig. 1). This cylinder was divided in two. One half was frozen at -20 °C for PTX analysis, the other half was formalin-fixed and paraffin-embedded for cleaved caspase-9 staining.

Collection of plasma samples

Blood was sampled in heparin-containing tubes via a catheter that was placed in the arteria carotis or arteria femoralis, and blood samples were taken between 5 min and 8 hours after starting the perfusion. After sampling, rats were euthanized. Blood samples were immediately centrifuged, and separated plasma was stored at -80 °C until analysis. The perfusate and plasma samples were analysed as described in Chapter 1.

For the bioavailability study, the PTX plasma concentrations were log transformed before analyses and were compared using a student *t*-test with a significance level of 0.05 (SPSS 20.0).

PTX tissue concentration

After rinsing and weighing, samples were suspended in water and tissue was mechanically disrupted. Subsequently, internal standard (C^{13}-paclitaxel) was added. Tumor tissue was then enzymatically digested using a proteinase-K and a lipase solution (both Sigma-Aldrich, Diegem, Belgium). Tissue suspensions were left to incubate overnight at 55 °C whilst continuously being shaken (IKA® Werke, Staufen, Germany).

After centrifugation of the digested tissue suspension at 10.000xg for 20 min, supernatant was collected and subjected to solid phase extraction. In short, after loading the sample, the SPE cartridges (Oasis® HLC cartridges, Waters, Etten-Leur, The Netherlands) were washed with a sodium hydroxide (pH 10.0) solution and a 70% (v/v) methanol solution in water. Following elution of PTX, samples were concentrated by evaporation of methanol under a mild nitrogen stream (30 min, 35 °C). Finally, after reconstitution, samples were injected onto the Waters Acquity UPLC system (BEH C18 column). Paclitaxel and C^{13}-paclitaxel were detected with a Quattro Ultima triple quadrupole system (Micromass Waters, Manchester, UK). The measurement range of the analytical method was 30.0 – 8000.0 pg/mg.

Given the observed non-linearity in the PTX penetration profile, only 1 side of the tumor was used to describe the penetration profile. The data was divided into 2 groups, the first group consisted of observations from the ventral part of the tumor, while the second group consisted of data from the dorsal part of the tumor. Overlap was allowed between both groups for measurements that were obtained in the centre of the tumor. After Ln-transformation of the measured PTX concentrations, the penetration could be approximated by a linear function of depth, using Statistical Analysis Software (SAS). In a second step, an elaborate mixed effects model, treating tumor size as a continuous predictor and time of measurement as a categorical predictor, was fit to the data.

Using likelihood-ratio tests (statistical test based on the goodness-of-fit of the model) the significance of the different model parameters was evaluated. Parameters that proved to be non-significant at the 5% level of significance were removed from the model.

Equation 1. Elaborate linear mixed effects model to predict the paclitaxel tumor concentration.

$$Ln(Conc)_{ijk} = Int + \beta_1 * time_i + \beta_2 * size_k + \beta_3 * Depth_j + \beta_4 * Depth_j * time_i + \beta_5 * Depth_j * size_k + \beta_6 * time_i * size_k + b_k * Depth_j + \varepsilon_{ijk}$$

With:

Int = Intercept, β = slope

i (time) = 5, 15, 30, 45, 60, 90, 120, 240, 480 min

j (depth) = 0 – 20 mm

k = rat specific

Determination of apoptosis by cleaved caspase-9 immunohistochemistry

Sections of 5 µm thickness were mounted on a rotary microtome (HM® 360, Microm, Walldorf, Germany) which were deparaffinized in xylene and rehydrated in a downgraded series of ethanol. After washing in PBS, heat-induced antigen retrieval was performed for 30 min in a citrate buffer (pH 6), after which the tissue slides were cooled down for 15 min at room temperature. The endogenous peroxidase activity was blocked for 15 min with 0.3% hydrogen peroxide (DAKO, Glostrup, Denmark). After washing steps, each section was blocked with a blocking solution Tris-Buffered saline 0.1% (TBST 5% normal goat serum) for 1 hour at room temperature. Primary antibody cleaved caspase-9 (Cell signaling Technology, Leiden, The Netherlands, 1/400 in PBST 5%) was incubated overnight at 4 °C. After washing, the tissue sections were incubated for 30 min at room temperature with a labeled polymer-horseradish peroxidase anti-rabbit secondary antibody (DAKO). The color reaction was developed using the chromogen 3,3-diaminobenzidine+ (DAB) for 10 min. After washing, the tissue sections were counterstained with Mayer's haematoxylin for 2 min. TBST 5% normal goat serum was used instead of primary antibody as negative control to exclude false-positive responses from nonspecific binding of the secondary antibody.

The number of apoptotic cell fragments present in each tissue section was expressed as a fraction of the total number of cells. The apoptotic index was determined by Optronics Color

digital camera (Olympus Corporation, Tokyo, Japan) and specialized software (Cell D Olympus Imaging Solutions, Münster, Germany). Ten regions of interest (ROI's) were chosen at random at a magnification of 200x. High necrotic regions were excluded from analysis.

For the apoptosis study, the depth of apoptosis was compared using a one-way ANOVA with a significance level of 0.05. The differences between the depth of the apoptosis, the contact time and dose were compared using a student *t*-test with a significance level of 0.05 (SPSS 20.0).

RESULTS AND DISCUSSION

The amount of PTX reaching the tumor nodule is a predictor for the effectiveness of the IPEC treatment. To correlate the obtained PTX concentration in the tumor with the cytotoxic effect, the apoptotic index (%) was measured by staining cleaved caspase-9 positive cells.

In a first step, the time course of the cleaved caspase-9 positive cells (Fig. 2) in IPEC-treated rats was examined. The rats were treated for 45 min with 30 mg PTX (in 125 ml 0.9% NaCl) and the apoptosis was evaluated at different time points. A significant increase in apoptosis was achieved between 4 and 36 hours after IPEC treatment. The highest degree of apoptosis was obtained 8 hours post-treatment (Fig. 3).

In all other experiments, the degree of apoptosis will be evaluated between 4 and 8 hours after IPEC treatment. To ensure the observed apoptotic effect was due to the use of PTX and not to Cremophor®EL, the surfactant used in Taxol®, which has a cytototic effect itself, a 45 min IPEC treatment was performed with a 2% Cremophor®EL solution (i.e. similar to the concentration used in diluted Taxol®). No significant differences in apoptotic effect were observed after Cremophor®EL treatment when compared with the non-treated group (Fig. 2).

To evaluate the PTX distribution in the tumor, PTX concentration was measured in 129 tumor slices of 29 rats. The number of tissue slices per rat ranged from 2 to 8. For 20 rats, measurements were made per slice, while for 9 rats the whole tumor was analysed. For reasons of uniformity all measurements were recalculated to describe the depth of the centre of the tumor. Imputation was used to account for the missing observations. For these rats, the

depth of the slices was obtained by assuming that the total size of the tumor equalled the mean size for tumors with the same number of slices harvested. The impact of these imputed values on the model parameter, will be assessed once the final model is established. Figure 4 shows the measured PTX concentrations as a function of the depth of penetration. The subjects in whom depth measurements were obtained through imputation are depicted via red continuous lines in the spaghetti plots. Figure 4 clearly shows that the measured PTX concentrations are low in the core of the tumor and higher at the exterior of the tumor (ventral and dorsal side of the tumor).

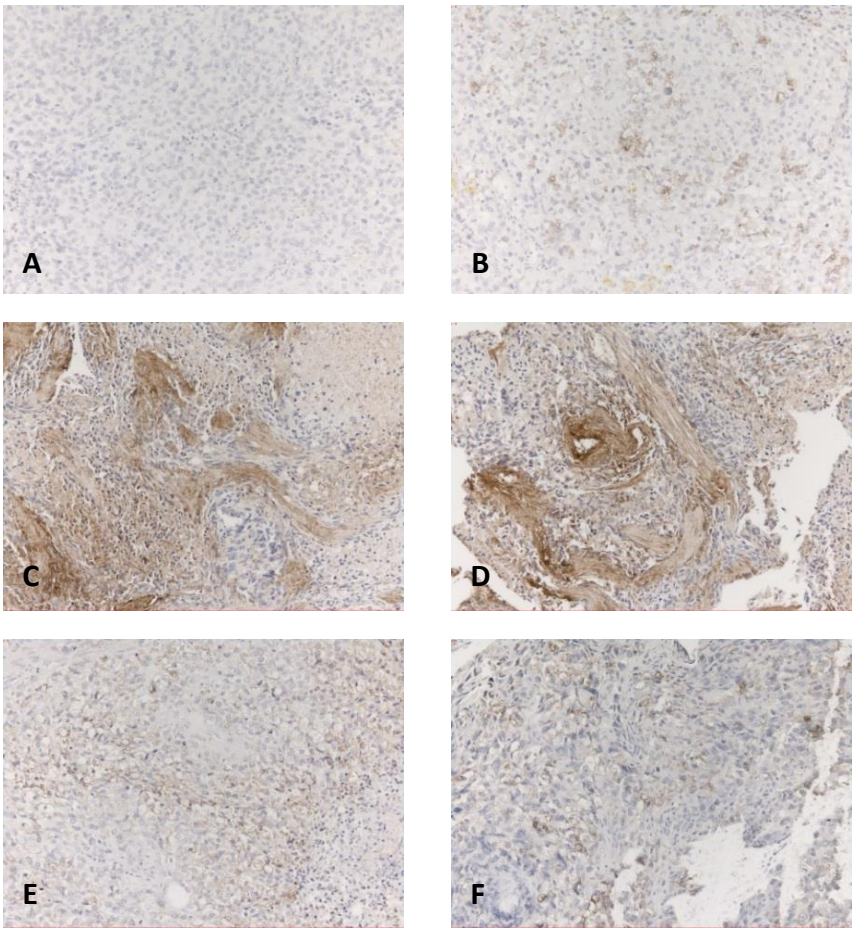

Figure 2. Cleaved caspase-9 immunostaining (x200 original magnification) of tumor specimens in the control group (A), and 1 hour (B), 4 hours (C), 8 hours (D), 48 hours after IPEC treatment with Taxol® (E) and 8 hours after IPEC treatment with 2% Cremophor®EL (F). Cells stained brown were positive for cleaved caspase-9 immunostaining.

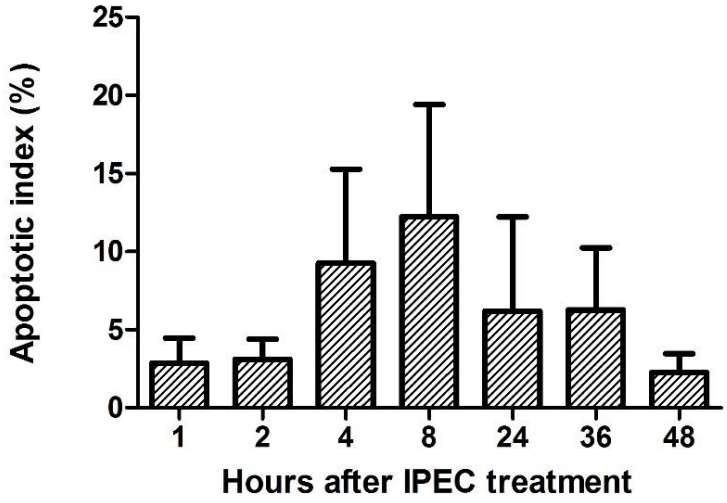

Figure 3. Time course of the cleaved caspase-9-positive cells (%) (± SD). IPEC treatment of 45 min with Taxol® (30 mg PTX).

The PTX concentration gradually decrease in function of depth, high PTX concentrations were observed up to 4 mm into the tumor (Fig. 5). Noteworthy is the fact that although one side of the tumor is connected to the abdominal wall, the PTX penetration at that side is similar to the PTX penetration at the side embedded in the abdominal cavity which is possibly due to the small connection between the tumor and the abdominal wall. No significant differences were observed in penetration depth between the dorsal and ventral side of the tumor. Which suggests that the PTX penetration is independent of the orientation of the tumor in the abdominal cavity. The PTX concentration is layer by layer uniformly distributed to the centre of the tumor. The size of the tumor does not influence the PTX penetration profile, as no significant differences were observed between the penetration profiles of tumors with different sizes ($p=0.080$). As in clinical practice visible tumors are removed by CRS, a PTX penetration of 4 mm will suffice to penetrate the complete residual tumor. The apoptosis study showed similar results as the degree of apoptosis is significantly higher in the outer layers of the tumor (0 – 4 mm) compared to the centre. Also no significant differences were observed between the apoptotic index of the ventral and dorsal tumor side (Fig. 6). The degree of apoptosis in the centre of the tumor is significantly higher than the baseline apoptotic signal (1.27% ± 0.06%) in untreated tumors confirming PTX penetration in the centre of the tumor.

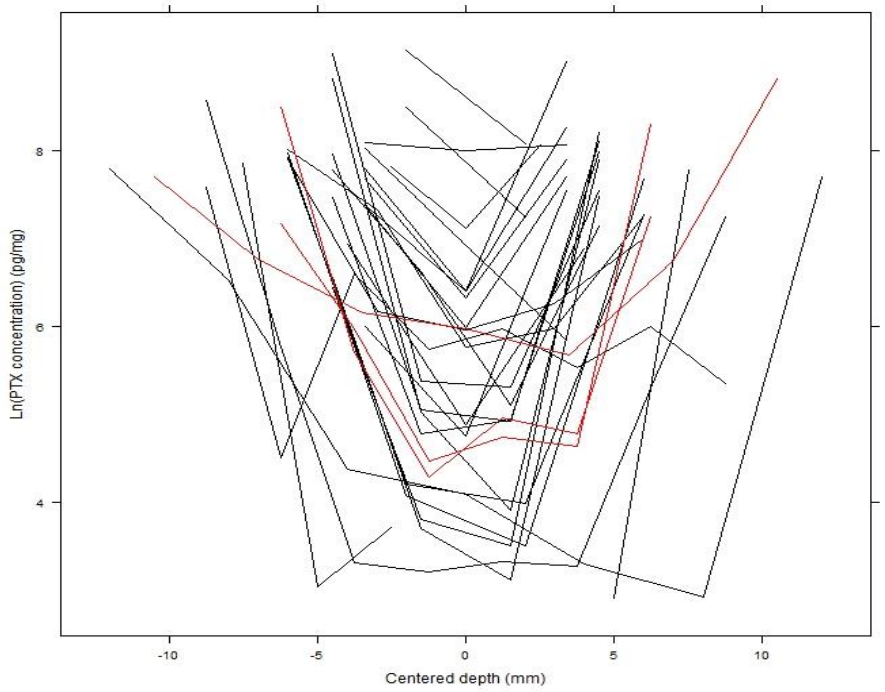

Figure 4. Spaghetti plot measured PTX concentrations as a function of the depth of excised tumor slice per rat. The red lines denote subjects for whom no size measurements were available and depth values were obtained through imputation.

The amount of PTX showing up in the tumor nodule is as much governed by non-pharmacokinetic variables (tumor nodule size, cell density, vascularity and interstitial fluid pressure) as by pharmacokinetic parameters (dose, duration, volume and carrier solution) [14]. On the basis of PTX tumor penetration, PTX plasma concentrations and the apoptotic index, an exploratory study was performed in order to characterize the tumor penetration, systemic pharmacokinetics (PK) and pharmacodynamics (PD) of PTX after IPEC.

The influence of the contact time on the PTX penetration profile was evaluated by removing the tumor at different time points. Unexpectedly, no increase in PTX concentrations was observed in the tumor by increasing the contact time. Nor were there differences observed in the PTX penetration profile. The PTX concentration remains unchanged between 5 min of treatment (first time point observed in the study) up to several hours post-treatment (Fig. 7A) indicating a saturable paclitaxel tumor penetration.

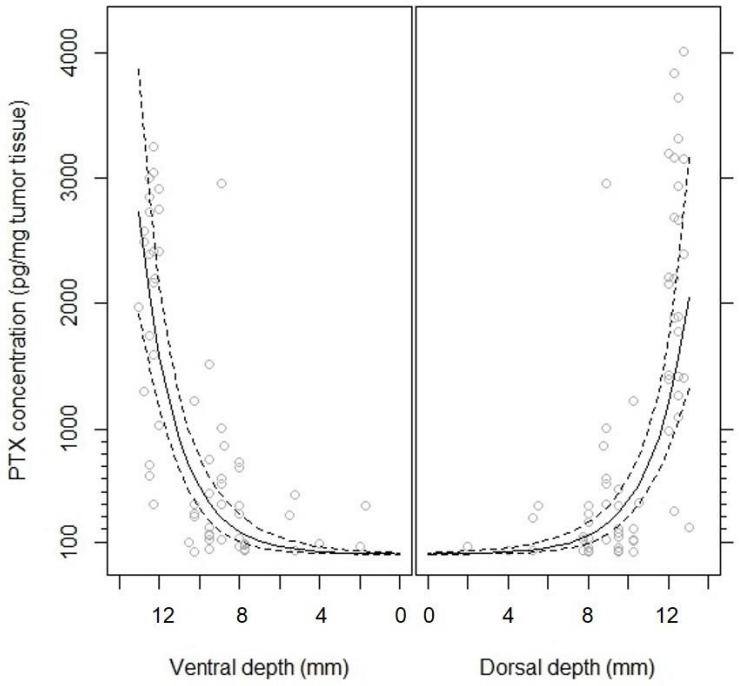

Figure 5. Scatterplots of observed paclitaxel concentrations as a function of penetration depth. The open circles depict the observations while the lines depict model predictions.

As the cytotoxic effect of PTX is related to the obtained concentration, it would be expected that the apoptotic index showed similar results after 15 min or 45 min of IPEC treatment. In contrast to this theory significant differences (p=0.00) in degree of apoptosis (6 ± 1% vs 12 ± 4%) were observed in the outer layers of the tumor (0 – 4 mm). A longer contact time resulted in similar intratumoral PTX concentrations but in an increased apoptotic effect (Fig. 8A). This may be explained by the fact that the efficacy of the drug ultimately depends on the intracellular concentrations. As both the intracellular and extracellular concentrations were measured, it is possible that the intracellular concentration is increasing by increasing the contact time. Although Jordan et al. (1996) described that for high extracellular concentrations (>0.001 mM), 30 mg PTX is equal to 0.28 mM, the saturation of the intracellular binding sites occurs almost readily in monolayer cultures [15-17]. In clinical practice, perfusions are performed from 30 min to 2 hours, on the basis of the developed PK-PD model (work in progress), it should be possible to predict the outcome of the increased treatment period.

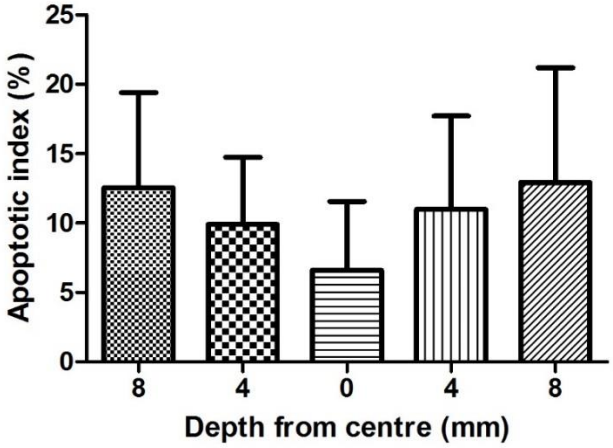

Figure 6. Measured apoptotic index (%) (± SD) as a function of tumor depth after IPEC treatment of 45 min with Taxol® (30 mg PTX).

The second analysed pharmacokinetic parameter was the effect of the dose on the PTX tumor and plasma concentrations. A dose of 3 mg PTX was compared to 30 mg PTX (i.e the MTD). The doses were used in a concentration of 0.024 mg/ml or 0.24 mg/ml. Although the concentration of the lower dose was 10-times lower than the MTD, the total PTX tumor concentration was similar compared to the high dose treatment (Fig. 7B).

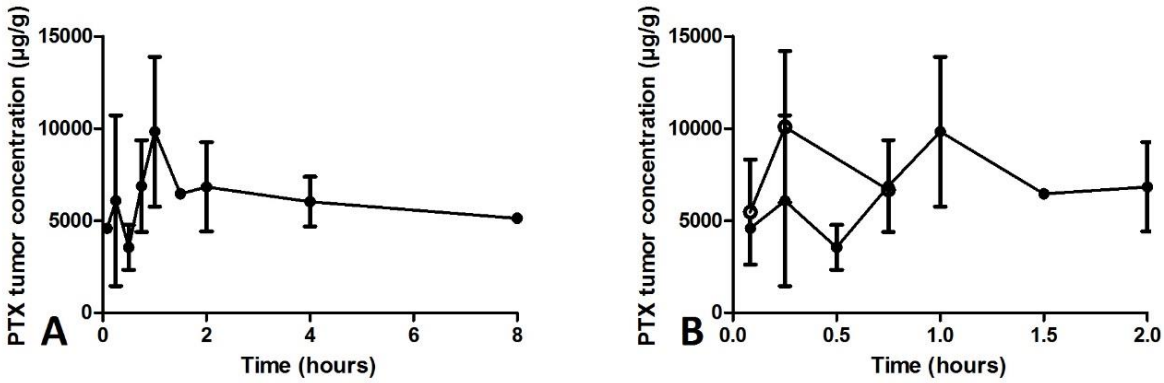

Figure 7. Paclitaxel tumor concentrations (µg/g) (± SD) in rats (n=3) after IPEC treatment with a paclitaxel dose of (●) 30 mg and (○) 3 mg in function of the time. (A) shows the PTX tumor concentration in function of time over a period of 8 hours, (B) focuses on the first 2 hours.

Also no differences were observed in the PTX tumor penetration profile compared to the used higher dose. Post-IPEC PTX tumor concentrations were not measured after low dose treatment. The apoptotic index was significantly lower (p=0.03) after low dose treatment (Fig. 8B) compared to the high dose treatment.

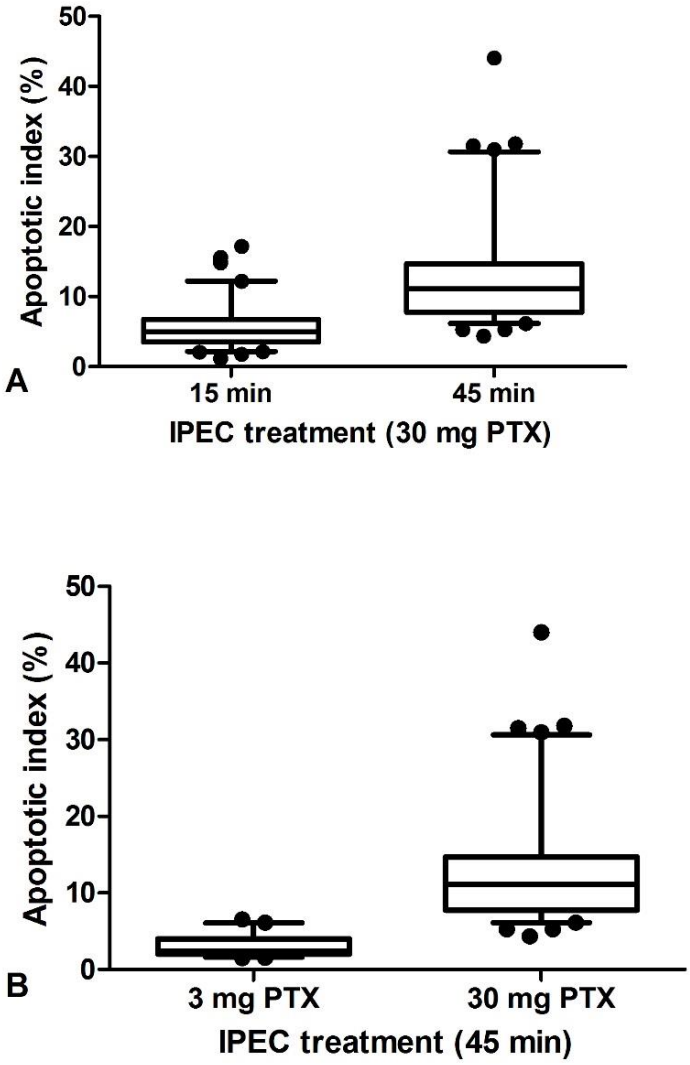

Figure 8. Apoptotic index (%) after IPEC treatment with variation in contact time (A), and variation in dose (B).

As well as the PTX tumor penetration, the PTX plasma concentrations were evaluated. The PTX plasma levels were much lower compared to the PTX tumor concentrations due to the presence of the peritoneal-plasma barrier. In contrast to the tumor concentrations, plasma concentrations are no value for the efficacy but are responsible for the systemic toxicity. Here,

differences between the low and high dose were observed (Fig. 9). Near the end of the IPEC treatment (i.e. 45 min after starting the procedure) the PTX plasma profiles are similar. The PTX plasma concentration is still increasing after removing the high dose perfusate, while after low dose treatment, the PTX plasma concentration immediately starts decreasing to lower concentrations. IPEC therapy with low dose PTX shows both an absorption and elimination phase, a C_{max} of 67 ng/ml was observed 45 min after starting the perfusion and a $T_{1/2}$ of 2 hours and 40 min was observed. The absorption after high dose perfusion gradually increased till 4 hours after starting the treatment. This suggests that the hydrophobic PTX might be stored in the other tissues, from which PTX is released after removing the chemotherapeutic liquid.

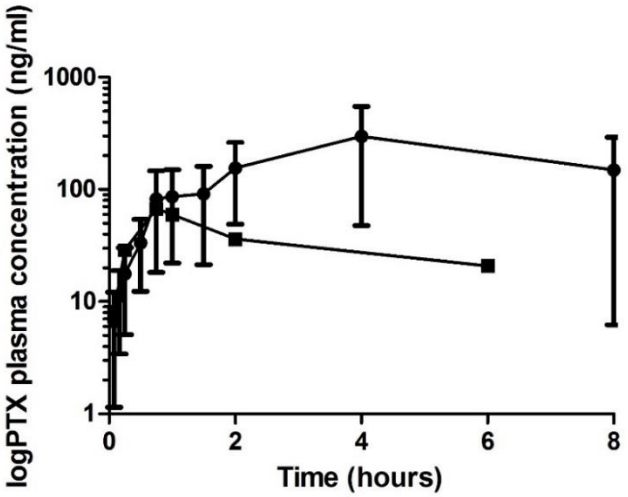

Figure 9. Paclitaxel plasma concentration (ng/ml) (± SD) in rats (n=3) after IPEC treatment (i.e. 45 min) with a PTX dose of (●) 30 mg and (■) 3 mg.

CONCLUSION

This study successfully illustrates how PTX is distributed in the tumor. In our xenograft tumor model, a PTX tumor penetration was observed independent of the size and the orientation of the tumor. Pharmacokinetic parameters as dose and contact time have an influence on the efficacy of the IPEC treatment but further research is necessary to get a complete picture of this process.

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CHAPTER 4

PHARMACOKINETIC EFFECTS OF PERFUSION TEMPERATURE, PERFUSION TIME AND DOSE AFTER IPEC WITH OXALIPLATIN

Manuscript of this chapter is in preparation:

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INTRODUCTION

For a long time, peritoneal carcinomatosis (PC) was considered a terminal condition. It is demonstrated that a complete cytoreductive surgery (CRS) to remove the macroscopic disease in combination with intraoperative intraperitoneal chemotherapy (IPEC) to eradicate microscopic residues enhances the overall survival of patients with PC [1]. Intraperitoneal chemotherapy needs to be performed immediately after surgery, to ensure that residual tumor cells do not get trapped in the postoperative fibrin adhesions [2]. The use of IPEC has been reported for PC from colorectal cancer (CRC), ovarian cancer (OC), pseudomyxoma peritonei (PMP), and malignant peritoneal mesothelioma [3]. The rationale for IP chemotherapy is based on the existence of a peritoneal-plasma barrier, which allows the usage of high local doses while limiting systemic drug exposure [4]. While high doses can be used, the limiting factor for IPEC treatment is the penetration of the cytotoxic drugs into the tumor tissue which is limited to only a few millimetres [5].

In contrast to the previous chapters PTX was not used as chemotherapeutic agent. In this clinical trial, oxaliplatin is used, as in our institution oxaliplatin is most commonly used for (H)IPEC treatment. Oxaliplatin, a diaminocyclohexane-platinum compound, which acts as an alkylating agent creating DNA adducts leading to apoptosis and impaired cell replication, is active against a variety of tumors such as CRC and OC [6]. In ovarian cancer, several large randomized trials have demonstrated a statistically significant survival increase associated with adjuvant IP platinum-based chemotherapy after primary CRS [7].

Although IPEC treatment under hyperthermic conditions (>41 °C) (HIPEC) is already performed in many institutions for many years, the additional effect of hyperthermia remains unclear and several conflicting results were published. Recently, it was shown that hyperthermia potentiates the local absorption of oxaliplatin in Sprague-Dawley rats and that hyperthermia would limit its systemic absorption [8]. On the other hand, hyperthermia also induces apoptosis in healthy cells which affects the healing of anastomosis [9]. Furthermore, Klaver et al. demonstrated no increase in survival rate using hyperthermia compared to normothermic treatement [10]. To our knowledge, a clinical benefit from hyperthermia combined with chemotherapy compared to chemotherapy alone was never demonstrated.

As is the case with the perfusion temperature, the duration of chemoperfusion has not yet been standardized. In literature perfusion times range from 30 min to 2 hours [11, 12]. The required perfusion time for oxaliplatin would be shorter compared to cisplatin or mitomycin-C which makes it more practical for clinical use [13]. However in vitro studies have shown that by increasing the contact time, the amount of cell death also increases [14]. In literature, the use of high dose oxaliplatin (460 mg/m^2) was first introduced for HIPEC for the treatment of PC of CRC [11]. Although the results are promising for high dose treatment, at the moment a variety of doses are used ranging from 200 mg/m^2 to 460 mg/m^2 [12].

In this study, two aspects of IPEC treatment were analysed. First, as the use of hyperthermia remains unclear, the use of hyperthermia in combination with oxaliplatin was compared to an oxaliplatin treatment alone in order to evaluate the possible synergistic effect of using hyperthermia. Second, the pharmacokinetic effect of varying the dose and perfusion time was evaluated by increasing the oxaliplatin dose from 200 mg/m^2 to 460 mg/m^2 while the perfusion time was decreased from 90 min to 30 min.

MATERIALS AND METHODS

Patient Selection

Data were obtained from 38 patients who were treated from May 2012 to March 2013 for primary or recurrent peritoneal carcinomatosis with cytoreductive surgery and (H)IPEC with oxaliplatin. Demographic data are illustrated in Table I. Written informed consent for (H)IPEC treatment was obtained prior to treatment, and approval of the study protocol was obtained from the institutional review board.

Chemoperfusion protocol

Surgery consisted of a combination of organ resection and peritonectomy procedures. Small bowel and ileocolic anastomoses were created before, and colonic anastomoses after the chemoperfusion. At the completion of the surgery, intravenous folate (20 mg/m^2) followed by 5-FU (400 mg/m^2) were administered to the non-ovarian cancer patients. Chemoperfusion

was performed using the open abdomen (coliseum) technique. Two Tenckhoff peritoneal catheters (Tyco Healthcare, Mechelen, Belgium) for infusion and three Jackson-Pratt drains (Cardinal Health Care, Dublin, Ohio, USA) for drainage were placed through the abdominal wall. Temperature probes were placed in the pelvis and left and right upper abdomen. The extracorporeal circuit consisted of a cardiotomy reservoir (Sorin Group, Mirandola, Italy), a CSC14 cardioplegia heat exchanger (Sorin Group, Mirandola, Italy), and a roller pump (COBE Cardiovascular, Denver, Colorado, USA). Upon completion of the circuit, a flow of approximately 1 L/min was instituted and the abdominal cavity was further heated to 37 °C or 41 °C depending on the treatment protocol.

Table I. Demographic details of (H)IPEC-treated patients with oxaliplatin.

	Group 1	Group 2	Group 3
Treatment parameters			
Dose	200 mg	460 mg	460 mg
Temperature	37 °C	37 °C	41 °C
Time	90 min	30 min	30 min
N	24	7	7
Age (years)	54.7 ± 14.2	53.0 ± 16.1	56.9 ± 8.3
Male (%)	25	14	28
Female (%)	75	86	72
Indication			
Colon cancer	7	7	4
Ovarian cancer	9	-	2
Neuroendocrine cancer	4	-	-
Gastric cancer	3	-	-
Other	1	-	1

Once the target temperature was reached, oxaliplatin (200 mg/m^2 or 460 mg/m^2) was added to the dextrose 5% solution (2 L/m^2) and circulated for 90 min or 30 min, respectively. Body surface area was calculated as the square root of (Weight (kg)*Length (cm))/3600). The patients were divided in three treatment groups: group 1 was treated at 37 °C for 90 min with

a dose of 200 mg/m^2 oxaliplatin, group 2 received a treatment at 37 °C for 30 min with a dose of 460 mg/m^2 and group 3 was treated at 41 °C for 30 min with a dose of 460 mg/m^2 oxaliplatin. Intra-abdominal temperatures were recorded before the start and every 5 min during chemoperfusion. The abdominal cavity was not rinsed with saline after the chemoperfusion procedure.

Sample collection

Blood and perfusion samples were collected during the chemoperfusion. For both 30 min and 90 min treatments perfusate and blood samples were taken just before starting the procedure and 1, 5, 10, 15, 20 and 30 min after starting the treatment. For the 90 min treatment, after 30 min, blood and perfusate samples were collected every 15 min till the end of the IPEC procedure. Postoperative blood samples were collected 2, 4, 8, 12, 24, 36 and 48 hours and 4 and 7 days after starting the treatment. Samples were kept at -20 °C until analysis by inductively coupled plasma-mass spectrometry (ICP-MS).

Tissue samples of the abdominal wall were collected after removing the cytotoxic agent from the abdominal cavity. Tissue samples were formalin fixed and paraffin embedded. Sections of 20 µm thickness were mounted on a rotary microtome (HM® 360, Microm, Walldorf, Germany) and kept on microscopic slides till analysis by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS).

Data collection

Postoperatively, the following parameters were collected: mortality (within 30 days or during hospital stay), major morbidity (defined as any undesired outcome, leading to prolonged hospital stay and/or reoperation), anastomotic leakage, major bleeding, reoperation rate, time to remove the nasogastric (NG) tube, duration of the hospital stay and grade 1 to 4 leukopenia and thrombopenia.

Platinum determination in blood and perfusate

Platinum (Pt) concentrations in blood and perfusate samples were determined using an Xseries 2 quadrupole-based inductively coupled plasma mass spectrometry (ICP-MS) instrument (Thermo- Scientific, Bremen, Germany). Sample introduction was accomplished via a concentric glass nebulizer mounted onto a Peltier-cooled conical spray chamber with impact bead. The peristaltic pump delivered the sample at a flow rate of 500 µl/min. A Cetac 500 autosampler (Cetac, Omaha, NE, USA) was deployed for automatic sample introduction. All the isotopes of Pt were monitored, along with the major isotope of thallium (205Tl). The instrument was operated at an RF power of 1.400 W. The nebulizer gas (argon) flow rate was maintained at 0.85 L/min. Five replicate measurements were performed for every sample and standard solution. Calibration solutions containing 0.25, 0.5, 1, 5, 10, 15, and 20 ng/g Pt were prepared and analysed. Correlation coefficients for the linear regression lines were always higher than 0.99.

Platinum determination in tissue samples

Tissue samples were analysed with laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). A New Wave Research UP193HE ArF* excimer-based laser ablation system (New Wave Research, Fremont, USA) coupled to an Element XR (Thermo Scientific, Germany) double-focusing sector field ICP-MS instrument was used to determine the distribution of Pt within the tissue sections. This LA-unit was equipped with a standard circular ablation cell with a diameter of 5 cm.

The area of interest was scanned with a laser beam with a diameter of 70 µm. The scanning parameters were selected such as to provide square pixels in the final elemental map [15]. The material released from the surface of the sample upon ablation was transported using helium as a carrier gas into the ICP ion source. Post-ablation, the helium flow carrying the dry sample aerosol was admixed with argon prior to introduction into the ICP. Both laser beam diameter and laser repetition frequency have been selected carefully, taking the nature of the sample and the concentration of the target element into account. Also the incident laser fluence has been optimized. The settings used for the LA-ICP-MS experiments are summarized in Table II.

Table II. Instrument settings and data acquisition parameters for LA-ICP-MS measurements.

Laser ablation	**New Wave Research**
Type	UP193HE ArF* excimer based laser
Measurements	Mapping
Wavelength (nm)	193
Lateral scanning speed (µm/sec)	80
Repetition frequency (Hz)	10
Laser energy (J/cm^2)	0.7-0.8
Diameter of laser beam (µm)	70
Ablation chamber	Standard cell
ICP-SFMS	**Element XR**
Mass resolution (m/Δm)	300
RF power (W)	850
Carrier gas flow rate (L/min)	Helium, 0.5
Make up gas flow rate (L/min)	Argon, 0.7
Number of runs	200-300
Number of passes	1
Time per pass (sec)	1

The obtained images were analysed by the NIH ImageJ software (version 1.39s, available from http://rsb.info.nih.gov/ij), the blue/green and red coloured pixels were counted and were presented as a fraction of the total amount of pixels of the image.

Statistics

Differences in the variables with a normal distribution were analysed with an unpaired student *t*-test, non-normally distributed variables were log-transformed before analysis. Statistical significance was assumed when $p \leq 0.05$. Data analysis was performed by means of SPSS 20.0.

RESULTS AND DISCUSSION

Although pharmacokinetics of oxaliplatin after IPEC treatment have been previously reported [16, 17], this study is unique as 2 different parameters for IPEC treatment were evaluated. On the one hand the influence of hyperthermia (41 °C) was evaluated during IPEC. It is known that hyperthermia has no influence on surgical outcome [18], but in a clinical setting, the influence of hyperthermia on pharmacokinetics and morbidity had yet not been investigated. On the other hand, the influence of dose and perfusion time on the pharmacokinetic parameters was compared using a high dose short treatment (460 mg/m^2 for 30 min) or a low dose long treatment (200 mg/m^2 for 90 min). It is not clear whether the dose or the perfusion time has the highest influence on oxaliplatin pharmacokinetics as 460 mg/m^2 for 30 min is the maximum tolerated dose in patients [11], while other studies have shown that treating cancer cells with oxaliplatin for 2 hours resulted in a twofold increase in cell death compared to a 30 min treatment [14].

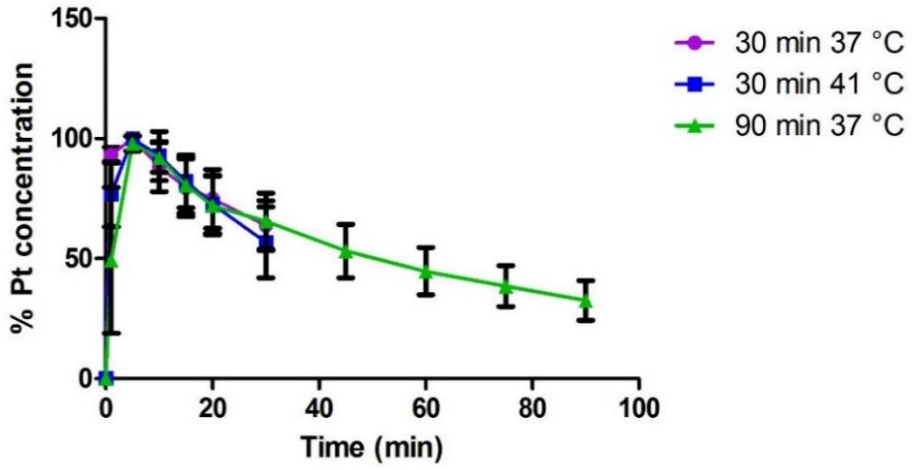

Figure 1. Mean (± SD) observed peritoneal platinum concentrations (initial dose=100%) for the different treatment groups.

First the influence of the 3 different treatment modalities on the Pt concentration in the perfusate in function of time was evaluated (Fig. 1). In the 3 groups, the Pt perfusate concentration decreased in function of time and no significant differences were observed between the different treatment groups, indicating that the percentual decrease of the Pt

concentration in the perfusate is independent of the treatment temperature or initial oxaliplatin dose. In literature, the half-life of Pt in the perfusate was between 18 and 42 min after starting the IPEC procedure [16, 17]. In our study, half of the administered dose was absorbed between 22 min and 72 min with a mean half-life of 57 min.

Influence of the temperature

In this part of the study, a selection bias should be taken in account, as the groups were not randomized and patients with the highest PCI score were automatically assigned to the hyperthermic treatment group. Comparing the mean Pt blood values and pharmacokinetic parameters (Table III), no significant differences were observed comparing the normothermic and hyperthermic-treated groups (Fig. 2). The peritoneal fluid to plasma AUC ratio is an important factor for the effectiveness of the IPEC treatment [19]. As no differences in Pt blood levels and Pt perfusate levels between the treatment groups were observed, the AUC ratio did not differ significantly (Fig. 3). When analysing the mean Pt blood values and pharmacokinetic parameters, high variances were observed in the hyperthermic-treated group (n=7).

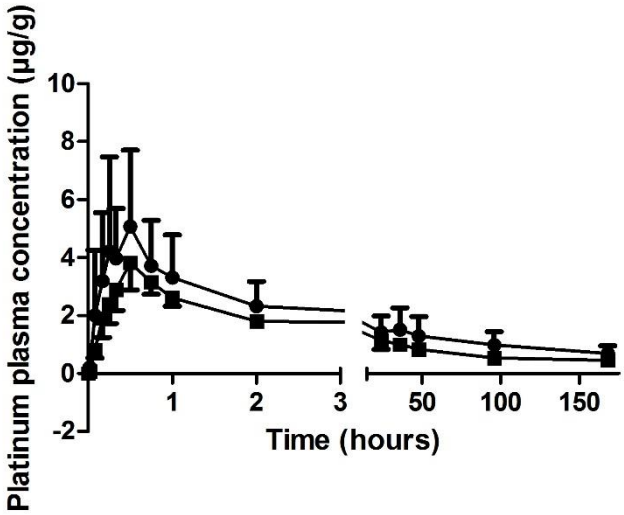

Figure 2. Platinum blood concentrations (µg/g) (± SD) in function of time over a period of 7 days in patients of (●) group 3 and (■) group 2.

Not all patients in this group depending on the extent of their disease received a complete CRS. These incurable patients received a hyperthermic oxaliplatin treatment as (semi-)

palliative treatment as it is known that hyperthermia has a cytotoxic effect by itself and it would increase drug penetration in solid tumors thereby increasing the cytotoxic efficiency [10, 20]. Next to these direct cytotoxic effects, hyperthermia would also enhance the antitumor effect of several cytostatic agents by increasing tumor blood supply and oxygenation [21].

Table III. Geometric pharmacokinetic parameters (± SD) of the different treatment modalities used during (H)IPEC.

Treatment groups	$AUC_{IP\ t=30min}$ (µg.h/g)	$AUC_{BI\ t=7days}$ (µg.h/g)	$AUC_{t=30min}$ ratio* (mean value)	C_{max} (µg/g)
Group 1	12.2 ± 0.9	111.4 ± 15.2	20.6	2.3 ± 0.6
Group 2	34.3 ± 4.3	129.6 ± 15.6	28.9	3.8 ± 0.9
Group 3	29.9 ± 8.2	158.0 ± 70.9	25.1	5.1 ± 2.4

*area-under-the-curve ratio of perfusate Pt concentration (AUC_{IP}) and blood Pt concentration (AUC_{BI}) over a period of 30 min

Although cytoreduction in hyperthermic-treated patients was less extensive compared to the normothermic-treated group more complications were observed in this group (Table IV). From the 7 patients treated with hyperthermia, 2 patients showed major bleeding after surgery, 1 patient had anastomotic leakage and 3 patients went back for surgery while none of the patients from the normothermic group showed these complications.

The World Health Organization (WHO) grading system classifies leukopenia and thrombopenia during chemotherapy as follows: absence (grade 0), mild (grade 1-2) and severe (grade 3-4). In the hyperthermic group (Group 3) 1 patient showed grade 3 leukopenia and 2 patients were diagnosed with thrombopenia (1 patient showed grade 1 and another grade 4 thrombopenia), while no patients in the normothermic group showed any bone marrow depression. Although both groups are small, these complications indicated a higher toxicity rate for the hyperthermic-treated group (Table IV).

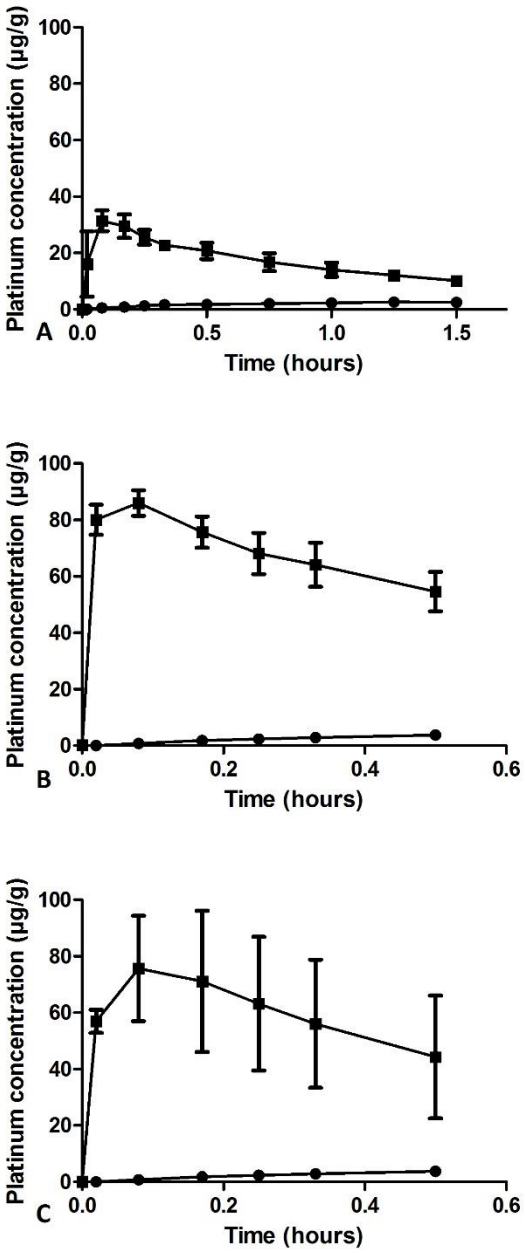

Figure 3. Platinum concentration (µg/g) (± SD) in (●) blood and (■) perfusate after IPEC treatment. (A) group 1, (B) group 2 and (C) group 3.

Although the penetration in healthy tissue is different compared to the penetration in tumor tissue due to its irregular cell structure and leaky blood vessels, the Pt penetration in healthy tissue is here a model for the penetration in tumor tissue. The Pt penetration in healthy tissue (abdominal muscle) was analysed as removing of all visible tumor tissue during CRS was preferred for the well-being of the patients. Figure 4 shows the results of the Pt penetration

through the abdominal muscle. The colours on the images represent the amount of Pt in the tissue, ranging from purple indicating very low concentrations over blue and green to red for very high Pt concentrations. Higher Pt concentrations were observed at the edge of the tissue in the hyperthermic-treated group, shown by the green coloured region (Fig. 4B).

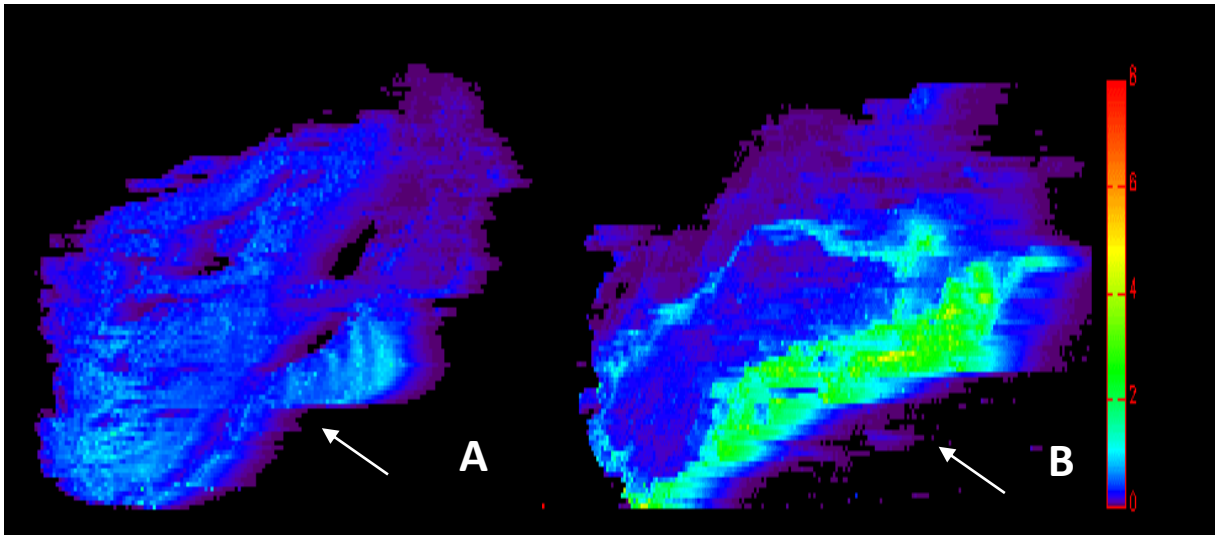

Figure 4. Platinum tissue penetration evaluated by LA-ICP-MS. (A) group 2, (B) group 3 (white arrow indicates the penetration side).

When analysing the complete tissue sample for the blue and green regions, no differences were observed. 29.3 ± 11.4% and 24.8 ± 13.2% of the pictures for the hyperthermic and normothermic-treated group, respectively, are coloured green or blue suggesting a similar overall Pt penetration. As the Pt concentration is slightly higher at the penetration side after HIPEC treatment, while centrally the Pt concentrations are similar, these images indicated that hyperthermia is not enhancing the Pt penetration and does not affect penetration depth. Thus, normothermic treatment resulted in a similar efficacy and a lower toxicity, which makes it a preferable treatment strategy considering the above mentioned complications with hyperthermic treatment.

Influence of the dose and perfusion time

The dose and perfusion time were evaluated with respect to their influence on oxaliplatin pharmacokinetics. Patients were divided in 2 groups: low dose long treatment (200 mg/m^2 for 90 min, group 1) and high dose short treatment (460 mg/m^2 for 30 min, group 2). Both groups

were treated under normothermic (37 °C) conditions. When comparing the Pt blood levels of both groups (Fig. 5) a significantly higher C_{max} (p=0.006) was observed for the patients treated with 460 mg/m^2 oxaliplatin. Nevertheless, the observed C_{max} in group 2 was lower compared to the observed values in literature after 2 hours intravenous infusion with clinically used doses of oxaliplatin whereby an acceptable systemic toxicity was determined [17].

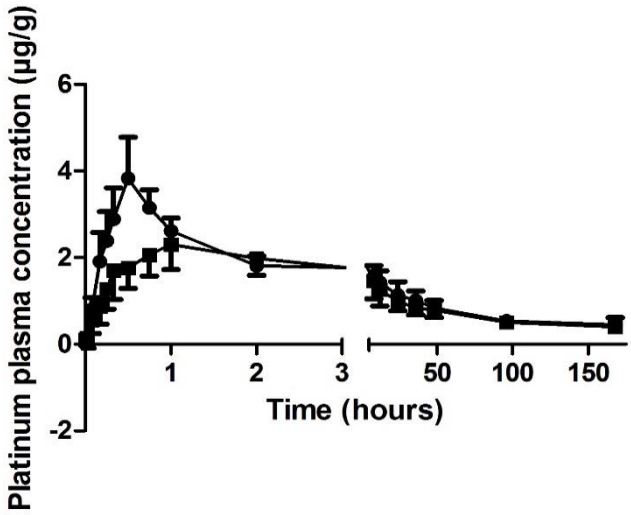

Figure 5. Platinum blood concentrations (µg/g) (± SD) in patients in function of time over a period of 7 days of (●) group 2 and (■) group 1.

In our study none of the patients of group 2 developed clinically important bone marrow depletion nor were any surgical complications observed (Table IV). C_{max} was reached 60 min and 30 min after starting the IPEC treatment, for group 1 and group 2 respectively. Although the perfusion in group 1 was performed during 90 min, the Pt blood concentrations already decreased before the end of the chemoperfusion, indicating that the Pt clearance is higher than the Pt absorption. These results can be related to the half-life of Pt in the perfusate (57 min). These findings would support a decrease of the perfusion time from 90 min to 60 min for IPEC treatment with 200 mg/m^2 oxaliplatin. In spite of the high C_{max} of group 2, the Pt blood levels decreased rapidly between 30 min and 60 min after starting the treatment, yielding the same blood concentrations as group 1 after 60 min. From that point on, the Pt clearance of both groups is similar (Fig. 5).

Regardless of the differences in the maximal concentration, no significant differences were observed for the $AUC_{BL,\ t=7days}$ (Table III). The AUC ratio for group 2 is significantly higher compared to the AUC ratio of group 1 (Table II), indicating that high Pt concentrations are delivered to the area of interest while the systemic toxicity is remains low.

Table IV. Observed complications after (H)IPEC treatment with oxaliplatin.

	Group 1	Group 2	Group 3
Surgery duration (h)	9.6 ± 2.6	8.6 ± 3.0	6.7 ± 3.1
Mortality	1	0	0
Major morbidity (%)	16.7	14.3	42.8
NG drainage (days)	5 (4 – 7.5)	6 (4 – 6)	4 (2.2 – 10.5)
Hospital stay (days)	14 (12 – 17)	12 (11 – 16.7)	13 (10.2 – 27.7)
Reoperation rate (%)	12.5	0	42.8
Major bleeding (%)	0	0	28.6
Anastomotic leak (%)	12.5	0	14.3
Leukopenia			
Grade 1	2	0	0
Grade 2	3	0	0
Grade 3	2	0	1
Grade 4	0	0	0
Thrombopenia			
Grade 1	5	0	1
Grade 2	1	0	0
Grade 3	0	0	0
Grade 4	0	0	1

These results were confirmed by the Pt tissue penetration study. Figure 6 clearly shows a significant difference between the Pt penetration of group 2 compared to group 1. Although the duration of the perfusion was 3-times higher, very low Pt concentrations were observed in patients treated with 200 mg/m^2. Only 2.7 ± 2.6% of the tissue coloured blue or green, while for the patients treated with 460 mg/m^2 for 30 min, 24.8 ± 13.2% of the tissue showed Pt penetration. Increasing the perfusion time did not result in a cumulative drug uptake. Most of

the cytotoxic drugs have a steep dose-effect curve [22] meaning that the cytotoxic effect is related to the delivered Pt concentration. Lower concentrations resulted in a lower cytotoxic effect. Based on these results, it can be concluded that the dose is an important factor for the Pt penetration while the contact time is of less importance.

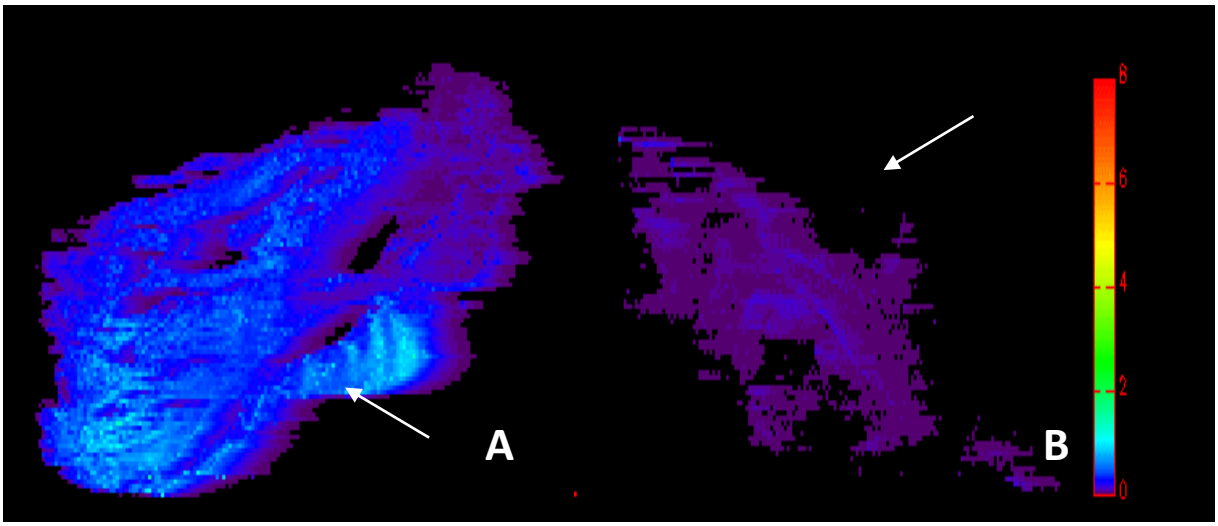

Figure 6. Platinum tissue penetration evaluated by LA-ICP-MS. (A) Group 2, (B) group 1 (white arrow indicates the penetration side).

CONCLUSION

As no additional effect of hyperthermia and a dose-related platinum penetration was observed, a high dose (460 mg/m^2) short normothermic treatment is preferable for IPEC treatment of peritoneal carcinomatosis from different origin.

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GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The main objective of this project was to evaluate different paclitaxel (PTX) formulations and different treatment modalities for intraperitoneal chemotherapy (IPEC) of peritoneal carcinomatosis of ovarian origin.

In a first step, we were able to develop a stable PTX nanocrystalline suspension containing PTX and Pluronic®F-127 in a ratio (w/w) of 4/1 by the wet milling technique. During this thesis, we have proven that all evaluated formulations (PTX nanocrystalline suspension, Abraxane®, Genexol®PM and Taxol®) were suitable for IPEC treatment. However, differences were detected in the toxicity of the different formulations. Although PTX tumor concentrations were similar for all formulations, Abraxane® had a higher efficacy compared to the other formulations, probably due to more recirculation of PTX to the tumor via the blood flow as higher PTX plasma concentrations were detected after Abraxane® treatment. As a low systemic exposure is preferred after IPEC treatment (i.e. resulting in a low toxicity) Genexol®PM, a biodegradable monomethoxy poly(ethylene glycol)-block-poly(D,L lactide) block copolymer, seems the most promising formulation for IPEC use.

During this project, we were able to map the penetration profile of PTX into a solid tumor. It was demonstrated that high PTX concentrations were found up to 4 mm inside the tumor, independent of the size and the orientation of the tumor in the abdominal cavity. Moreover, the PTX concentration was cell layer by cell layer uniformly distributed to the centre of the tumor.

The evaluation of different parameters (perfusion temperature, perfusion time and dose) yielded interesting findings: while in several institutions IPEC is performed under hypertermic conditions (>41°C), this work showed (via an experimental study with PTX and a clinical trial with oxaliplatin) that there is no clinical evidence that hyperthermia increases the outcome of the therapy: hyperthermia resulted in a similar efficacy and a higher toxicity compared to normothermic treatment (37 °C).

The clinical trial with oxaliplatin clearly showed that treating the patients with the maximum tolerated dose (460 mg/m^2) for a short period (30 min) had a similar toxicity compared to a low dose treatment (200 mg/m^2 for 90 min) in combination with a higher efficacy. The experimental study with PTX showed that reducing the exposure time from 45 min to 15 min had no influence on the penetrated PTX concentration, whereas it resulted in a lower efficacy based on the degree of apoptosis. Decreasing the PTX concentration from the maximum tolerated dose to a 10-fold lower dose had no influence on the PTX tumor concentration, while a lower degree of apoptosis was observed. The results obtained when varying the PTX dose and contact time indicated that PTX uptake in the tumor is saturable and occurs very fast. However, further research is required to elucidate the dose-effect relationship of PTX after IPEC treatment.

We can expect that IPEC for the treatment of peritoneal carcinomatosis becomes the new standard of care, however unresolved issues remain. In this study just a few parameters of the IPEC process were evaluated. Despite the extensive use of IPEC for ovarian cancer since its first report, the optimal perioperative IP chemotherapy has not yet been established. Other parameters such as volume, novel cytotoxic agents and their dose, are still unexplored. To optimize and to gain a complete insight into the IPEC process, all parameters should be evaluated. A pharmacokinetic model should be developed which makes it possible to predict the outcome of the applied parameters. Afterwards these parameters should be evaluated in randomized trials, based on the results of these experimental data and the developed PK/PD model. The need to perform more randomized trials has been noted for several years. For the moment 3 large, randomized trials are ongoing in the Netherlands, France and Italy. These studies will help to answer questions about the role of IPEC following neoadjuvant chemotherapy, the efficacy of IPEC and the morbidity typical for IPEC. However these studies do not provide answers on the questions concerning the different parameters used during IPEC.

Poor drug penetration into tumor tissue remains an important factor. This penetration issue is attributed to the high interstitial fluid pressure caused by vascular hyperpermeability and the lack of functional lymphatics [1]. While traditionally chemotherapy is chosen in function of the tumor type, a new treatment strategy is that the tumor is adjusted to the

chemotherapy. For example, this is possible by using angiogenesis inhibitors (for example bevacizumab, Avastin®), which would normalise the vasculature and reduce the interstitial fluid pressure. For IV therapy, it is already proven that by pretreatment with angiogenesis inhibitors the chemotherapy reaches the tumor in higher concentrations. The same would be possible for IP treatment. The promising data emerging from regional administration of chemotherapy has prompted interest and investigation into the IP administration of non-cytotoxic agents with antineoplastic effects. Areas of active investigation include the regional instillation of gene vectors, immunotherapy and monoclonal antibodies [2].

From a pharmaceutical point of view, research should be performed to develop new formulations specifically designed for IP treatment as current IP treatment relies on the off-label use of products developed for IV applications. The main goal for IP drug delivery systems is to maintain a high local drug concentration within the peritoneal cavity, whereby drug delivery systems which remain in the peritoneal cavity for a longer period and release their drug slowly over a prolonged period are the most promising systems. Another major concern after cytoreductive surgery (CRS) is the formation of peritoneal adhesions. During the removal of the tumor nodules, peritoneal defects are created. These peritoneal defects are the basis in the development of peritoneal adhesions. Peritoneal adhesions cause abdominal pain, are ideal places for new tumor development and they affect postsurgical IP chemotherapy by impeding a uniform drug distribution in the abdominal cavity [3, 4]. Thus, drug delivery systems, such as hydrogels, should be developed, which release their drug slowly and also prevent the development of peritoneal adhesions after surgery. To evaluate this new hydrogels a new animal model should be developed, to evaluate both the anti-tumor effect and the prevention against peritoneal adhesions.

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SUMMARY

Ovarian cancer is the second most common gynaecological malignancy. As the symptoms of ovarian cancer are vague (e.g. abdominal pain, bleeding and abdominal swelling), most patients already show dissemination on the peritoneum, called peritoneal carcinomatosis, at the time of diagnosis. Advanced stage ovarian cancer remains confined to the peritoneal cavity for a prolonged period of time which makes it a good candidate for regional cancer treatment. Lately, the new standard of care consists of removing all visible disease by extensive surgery, followed by (hyperthermic) intraoperative intraperitoneal chemotherapy ((H)IPEC). Although IPEC has already been performed for many years in the specialized centres, no standardized treatment design has been developed in terms of schedule, residence time, drug, or carrier solution. Therefore, the aim of this project was to evaluate different parameters such as perfusion temperature, cytotoxic formulation and dose and the perfusion time used during IPEC treatment.

Paclitaxel (PTX), a good candidate for IPEC treatment due to its preferred pharmacokinetic profile, is not often used because of the side effects caused by Cremophor®EL, the solubilizer used in the commercially available PTX formulation, Taxol®. Therefore, in **Chapter 1** a new nanocrystalline PTX formulation suitable for HIPEC was developed with Pluronic®F-127 as single additive to overcome the systemic side effects. A stable PTX nanocrystalline suspension (± 400 nm) was prepared via the wet milling technique (60 hours at 150 rpm) obtaining a PTX/Plu F127 nanosuspension (4/1 ratio). In vitro cytotoxicity showed a lower toxicity for Pluronic®F-127 compared to Cremophor®EL, but a similar cytotoxicity for the 2 formulations. The in vivo toxicity of the PTX nanosuspension after HIPEC treatment was evaluated by determining the maximum tolerated dose (MTD) and evaluating the bioavailability. The effect on tumor growth was evaluated by Magnetic Resonance Imaging (MRI) at day 7 and 14 after HIPEC treatment in rats with peritoneal carcinomatosis of ovarian origin. The MTD of this nanosuspension was similar compared to Taxol® (30 mg PTX in 125 ml 0.9% NaCl). The PTX plasma levels were monitored for 90 min (i.e. 45 min of HIPEC treatment and 45 min post-treatment), and showed that during HIPEC treatment the PTX plasma levels were similar for both formulations. After treatment the PTX levels were increasing for the nanosuspension-

treated rats, while the PTX concentration remained constant for the rats treated with Taxol®. This effect can be explained by the adhesion of the nanoparticles to the intra-abdominal mucosa whereby a prolonged release of PTX was created. This approach overcomes one of the limitations of conventional intraperitoneal therapy where drugs are rapidly cleared from the peritoneal cavity. In spite of this advantage no significant differences on tumor growth were observed between Taxol® and the nanosuspension-treated group. However, the MRI data showed a significant reduction of tumor volume after HIPEC treatment with the PTX nanosuspension compared to the non-treated group.

In **Chapter 2** the added value of hyperthermia (41.5 °C) in combination with different PTX formulations (Taxol®, Abraxane® and Genexol®PM) for intraperitoneal chemotherapy (IPEC) was investigated in a human ovarian SKOV-3 xenograft tumor model in rats. IPEC treatment is characterized by high local doses resulting in a high efficacy and a low systemic toxicity. Therefore, for all treatment modalities both the efficacy and toxicity were evaluated. The toxicity of the treatment modalities was evaluated by determining the maximum tolerated dose (MTD), recovery time and bioavailability. The efficacy of the different treatment protocols was evaluated by a tumor growth delay study (TGD) and by analysing the PTX concentration in the tumor. The additional effect on the tumor growth of hyperthermia in combination with the PTX formulations was evaluated using a LAPLACE estimation method in NONMEM®. The MTD of Genexol®PM (0.27 mg PTX/ml) after HIPEC treatment was higher compared to the MTD of Abraxane® and Taxol® (0.24 mg PTX/ml). A faster recovery was observed after normothermic treatment with Abraxane® and Genexol®PM compared to hyperthermic treatment and treatment with Taxol® (both hyperthermic and normothermic). The bioavailability study showed higher PTX plasma concentrations for Abraxane® compared to Taxol® and Genexol®PM. No significant differences in bioavailability were observed between normothermic and hyperthermic conditions. Compared to the non-treated group tumor volume decreased after IPEC treatment for all treatment modalities. Although the PTX concentration in the tumor was independent of the formulation or temperature, (H)IPEC treatment with Abraxane® showed a higher decrease in tumor volume compared to Taxol® and Genexol®PM. This can suggest that the effect of Abraxane® is determined by both the PTX tumor penetration and the recirculation of PTX. No significant decrease in tumor volume was observed between the different temperatures. Hyperthermia gives no added value to IPEC

treatment as a higher toxicity and an equal efficacy was observed compared to normothermic treatment.

The tumor penetration of a cytotoxic drug after IP therapy is a limiting factor for treatment efficacy. Limited information is available about the PTX penetration after IP administration. In **Chapter 3**, the penetration profile of PTX into the tumor was examined and the efficacy of this penetration was evaluated by scoring the apoptosis. In a second phase, an exploratory study was performed in order to characterize the tumor penetration, systemic pharmacokinetics (PK) and pharmacodynamics (PD) of PTX after IPEC (by varying the contact time and dose of the treatment). In this study, Taxol® was used as PTX formulation. To evaluate the PTX tumor concentration and the apoptosis in function of time, a biopsy of the tumor was taken at different time points. One half of the biopsy was used for PTX analysis by UPLC-MS, the other half was formalin-fixed and paraffin-embedded for the apoptosis study by cleaved caspase-9 staining. The measured PTX concentrations gradually decreased in function of tumor depth, and high PTX concentrations were observed up to 4 mm into the tumor. No differences in PTX concentrations were measured between the side of the tumor connected to the abdominal wall and the tumor side embedded in the abdominal cavity, meaning that PTX is uniformly distributed in the tumor cell layer by cell layer. Similar results were found in the apoptosis study as the highest degree of apoptosis was observed in the outer layer of the tumor. Varying the contact time or lowering the PTX dose had no influence on the PTX penetration profile. The penetration profile was not affected when the treatment time was varied between 5 min up to several hours post-treatment. Although the intratumoral PTX concentrations are similar, differences in degree of apoptosis were observed. While a high degree of apoptosis (12 ± 4%) was observed after IPEC treatment of 45 min with 0.24 mg/ml PTX, only a low amount of apoptosis was observed when lowering the contact time (6 ± 1%) or using a dose of 0.024 mg/ml PTX (4 ± 1%).

In **Chapter 4** the pharmacokinetics of the perfusion temperature, perfusion time and the dose were evaluated in a clinical trial using oxaliplatin as cytotoxic agent. 38 patients were treated for primary or recurrent peritoneal carcinomatosis with cytoreductive surgery and (H)IPEC. The patients were divided in 3 different (H)IPEC treatment groups: 90 min 200 mg/m^2 oxaliplatin treatment at 37 °C (group 1), 30 min 460 mg/m^2 oxaliplatin treatment at 37 °C

(group 2) and 30 min 460 mg/m^2 oxaliplatin treatment at 41 °C (group 3). The amount of platinum (Pt) in the perfusate and blood was determined by inductively coupled plasma-mass spectrometry (ICP-MS), and platinum tissue penetration was analysed by laser ablation ICP-MS (LA-ICP-MS). Postoperatively, morbidity and mortality were also evaluated. Comparing the normothermic and the hyperthermic-treated groups (group 2 versus group 3) no significant differences in pharmacokinetic parameters were observed. Also no differences were observed in the Pt tissue penetration profile. Nevertheless, the clinical data showed an increased systemic toxicity for patients treated under hyperthermic conditions. Comparing a short high dose treatment (group 2) with a long low dose treatment (group 1), both under normothermic conditions, showed differences in the pharmacokinetic profile. The C_{max} was significantly higher for patients in group 2, while the $AUC_{t=7days}$ was similar for both groups resulting in a similar systemic toxicity. Also the tissue penetration was much higher for the patients of group 2 compared to patients of group 1 (24.8 ± 13.2% versus 2.7 ± 2.6%). These data indicated that the dose is an important factor for IPEC treatment while the contact time is of less importance.

In summary, it was clearly shown that hyperthermia had no additional effect on IPEC treatment and that the used dose is a more important factor compared to the perfusion time. Genexol®PM, Abraxane® and PTX/Plu F127 nanosuspension (4/1 ratio) are suitable formulations to replace Taxol® for the treatment of peritoneal carcinomatosis of ovarian origin. In this context future research should be focussing on optimizing IPEC therapy by evaluating the different parameters and developing of a pharmaceutical formulation which releases its cytotoxic agent for a prolonged period.

SAMENVATTING

Ovarium of eierstokkanker is de tweede meest voorkomende gynaecologische kanker bij Europese vrouwen. Omdat de symptomen bij eierstokkanker, steeds terugkerende buikpijn, licht vaginaal bloedverlies en een opgeblazen gevoel, vaag zijn en pas laattijdig optreden, worden bij de meeste patiënten reeds uitzaaiingen op het buikvlies (peritoneale metastasering) vastgesteld op het moment van de eerste diagnose. De uitzaaiingen bij gevorderde ovariumkanker (stadium III en IV volgens de FIGO richtlijnen) blijven meestal beperkt tot de buikholte, verdere uitzaaiingen naar de longen, de lever of andere organen komen niet vaak voor. De behandeling van buikvlieskanker bestaat erin de zichtbare kanker nodules chirurgisch te verwijderen. Om te voorkomen dat de overblijvende losse en niet-zichtbare kanker cellen zich opnieuw gaan inplanten en uitbreiden is er de laatste jaren een techniek ontwikkeld waarbij de buik tijdens de operatie gespoeld wordt met (verwarmde) chemotherapie ((H)IPEC). Hoewel (H)IPEC al sinds eind de jaren ‘90 wordt uitgevoerd in de gespecialiseerde centra, bestaat er nog steeds geen algemene behandelingsmethode die de behandelingsduur, het gebruik van de verschillende cytotoxische geneesmiddelen en het gebruikte vehiculum beschrijft. Tijdens deze studie werden verschillende parameters zoals de behandelingstemperatuur, de gebruikte farmaceutische formulatie, de gebruikte dosis en de perfusietijd tijdens IPEC geëvalueerd.

Paclitaxel (PTX) is door zijn goede farmacokinetische eigenschappen een uitstekende kandidaat als cytotoxisch geneesmiddel voor (H)IPEC behandelingen. PTX wordt echter niet vaak gebruikt omdat Cremophor®EL, het excipiënt aanwezig in het commercieel verkrijgbare Taxol®, allergische reacties kan veroorzaken. Om deze nevenwerkingen te voorkomen, werd er in **Hoofdstuk 1** een nieuwe nanokristallijne PTX formulatie ontwikkeld voor het gebruik bij (H)IPEC behandelingen met Pluronic®F-127 als enige excipiënt. Via de wet milling techniek, werden PTX en Pluronic®F-127 60 uur aan 150 tpm gemalen, waardoor een stabiele nanosuspensie (± 400 nm) werd verkregen met een PTX/Plu F127 ratio van 4/1. De cytotoxiciteit van Taxol®, de PTX/Plu F127 nanosuspensie en hun excipiënten werd in vitro getest.

Hoewel de cytotoxiciteit van Pluronic®F-127 lager was dan deze van Cremophor®EL was de cytotoxiciteit van beide formulaties vergelijkbaar door het effect van PTX. Een goede formulatie voor (H)IPEC behandelingen wordt gekenmerkt door een hoge lokale geneesmiddel concentratie in combinatie met een lage systemische toxiciteit. De toxiciteit van de PTX nanosuspensie en Taxol® na een HIPEC behandeling werd geëvalueerd door het bepalen van de maximum tolereerbare dosis (MTD) en het evalueren van de biologische beschikbaarheid. Het effect van de formulaties werd geëvalueerd door de evolutie van de tumorgroei (TGD) via magnetische resonantie (MRI) op te volgen na HIPEC behandeling. Het initiële tumorvolume werd bepaald op dag 0, de dag nadien, op dag 1, ondergingen de ratten een HIPEC behandeling waarvan het effect aan de hand van het tumorvolume werd bepaald op dag 7 en dag 14.

De MTD was voor beide formulaties (Taxol® en de PTX nanokristallijne formulatie) gelijk aan 30 mg PTX opgelost in 125 ml 0.9% NaCl. De PTX plasma waarden werden gevolgd over een periode van 90 min, concreet wil dit zeggen dat de plasmawaarden werden opgevolgd tijdens de 45 min durende HIPEC alsook de eerste 45 min post-operatief. De PTX plasmaconcentraties waren tijdens de behandeling voor beide formulaties vergelijkbaar. Na het verwijderen van de cytotoxische Taxol®-oplossing bleven de PTX spiegels constant, terwijl de PTX levels in het plasma bleven stijgen na de behandeling met de nanokristallijne formulatie. Dit kan verklaard worden door het feit dat de nanopartikels zich vasthechten op de mucosa vanwaar ze geleidelijk worden vrijgesteld. Op deze manier wordt er een verlengde PTX afgifte verkregen. Deze verlengde vrijstelling zorgt ervoor dat één van de limiterende factoren van HIPEC, de snelle klaring van het geneesmiddel uit de buikholte via de lymfevaten, wordt overwonnen. Ondanks dit grote voordeel werden er geen verschillen tussen beide formulaties waargenomen in tumorvolume na de HIPEC behandeling. Wel was er een procentuele daling in het tumor volume voor beide formulaties in vergelijking met de niet-behandelde ratten.

In **Hoofdstuk 2** werd de toegevoegde waarde van hyperthermie (41.5 °C) in combinatie met verschillende PTX formulaties (Taxol®, Abraxane® en Genexol®PM) voor intraperitoneale chemotherapie in een SKOV-3 xenograft rat model geëvalueerd. Het grote voordeel van IPEC is dat er hoge dosissen chemotherapie kunnen gebruikt worden waardoor een hoger effect (meer celdood) kan bereikt worden maar door de aanwezigheid van de peritoneale-plasma

barrière blijft de systemische toxiciteit beperkt. De toxiciteit van de verschillende behandelingsgroepen werd geëvalueerd via een MTD studie, het herstel van het lichaamsgewicht na IPEC en de PTX plasma concentraties. Het effect van de behandeling werd nagegaan aan de hand van de evolutie van de tumorgroei alsook door het bepalen van de hoeveelheid gepenetreerde PTX in de tumor. De MTD van Taxol® en Abraxane® na een HIPEC behandeling werd vastgelegd op 0.24 mg/ml terwijl voor Genexol®PM een MTD van 0.27 mg/ml werd bepaald. Het gewicht van de ratten was sneller genormaliseerd na een normotherme behandeling (37 °C) met Abraxane® of Genexol®PM terwijl hogere PTX plasma spiegels werden vastgesteld voor Abraxane® in vergelijking met Taxol® of Genexol®PM. Er werden geen significante verschillen in biologische beschikbaarheid vastgesteld tussen de normotherme en de hypertherme behandelingen. Het tumor volume daalde na behandeling met alle PTX formulaties in vergelijking met de niet-behandelde groep. Behandeling met Abraxane® gaf een grotere daling in tumor volume in vergelijking met een behandeling met Genexol®PM en Taxol®. De hogere PTX plasma spiegels na een IPEC behandeling met Abraxane® kunnen niet enkel leiden tot een hogere toxiciteit maar zouden er ook voor kunnen zorgen dat de hoeveelheid PTX die terugkeert naar de tumor (recirculatie) groter is, waardoor dit mechanisme ook inwerkt op de inhibitie van de tumorgroei. Een hypertherme behandeling had geen extra invloed op de procentuele daling in tumor volume. In de tumor werden hoge PTX concentraties gevonden onafhankelijk van de gebruikte formulaties of temperatuur. Uit deze resultaten kan er geconcludeerd worden dat hyperthermie geen toegevoegde waarde heeft voor het gebruik bij IPEC behandelingen. Na een HIPEC behandeling is de toxiciteit duidelijk hoger terwijl er geen significante verbetering in effect waarneembaar is. Uit deze studie blijkt dat zowel Genexol®PM en Abraxane® goede formulaties zijn om Taxol® te vervangen bij IPEC behandelingen.

Uit verschillende studies blijkt dat de penetratie van het cytotoxisch geneesmiddel in de tumor na een IP behandeling de limiterende factor is. Over de penetratie van PTX in de tumor bestaan er nog heel wat onduidelijkheden. Daarom werd in **Hoofdstuk 3** het penetratieprofiel van PTX opgesteld. Het effect van de PTX penetratie werd geëvalueerd door het scoren van de apoptose. In een tweede fase werd een preliminaire studie uitgevoerd waarbij aan de hand van de tumorpenetratie de farmacokinetiek (PK) en de farmacodynamiek (PD) van PTX na IPEC werd geanalyseerd. Dit gebeurde door de behandelingsduur en de toegediende dosis te

variëren. In de volledige studie werd Taxol® gebruikt als PTX formulatie. Om de evolutie van de PTX concentratie en de hoeveelheid apoptose in de tumor te evalueren in functie van de tijd, werd er een biopsie van de tumor genomen op verschillende tijdpunten bij verschillende ratten. Deze cilinder werd verdeeld in twee helften, de ene helft werd diepgevroren, hiervan werd de PTX concentratie bepaald door UPLC-MS, de andere helft werd gefixeerd in formol en daarna ingebed in paraffine. Deze geparaffineerde coupes werden gebruikt voor de apoptose studie. De geobserveerde PTX concentraties in de tumor daalden gradueel in functie van de diepte. Concreet werden er hoge PTX concentraties gevonden tot 4 mm diepte. De oriëntatie van de tumor in de buikholte speelde geen rol, de concentraties die gevonden werden aan de zijde van de tumor die in rechtstreeks contact stond met de cytotoxische oplossing zijn even groot als de concentraties gevonden aan de zijde die verbonden was met de buikspier. Dit toont aan dat PTX per cellaag homogeen verdeeld is in de volledige tumor. De apoptose studie bevestigt deze resultaten. De graad van apoptose was veel hoger in de buitenste (cel)lagen van de tumor en meer naar het centrum van de tumor daalde de apoptose. Een kortere behandelingsduur of het toedienen van een lagere dosis PTX had geen invloed op de hoeveelheid PTX die penetreerde in de tumor. Hoewel de intra-tumorale concentraties niet varieerden, waren er wel duidelijke verschillen in graad van apoptose zichtbaar bij het variëren van de perfusietijd of PTX dosis. Bij de standaard behandeling (0.24 mg/ml PTX en een perfusietijd van 45 min) was er 12 ± 4% van de cellen in apoptose, terwijl na een korte behandeling van 15 min slechts 6 ± 1% van de cellen apoptotisch waren. Het verlagen van de dosis tot 0.024 mg/ml leverde een apoptose van 4 ± 1% op.

In **Hoofdstuk 4** werd tijdens een klinische studie het effect van de perfusie temperatuur, de perfusietijd en de gebruikte dosis op de farmacokinetiek van oxaliplatine nagegaan. In deze studie werden 38 patiënten met peritoneale uitzaaiingen van verschillende primaire oorsprong behandeld met een cytoreductive chirurgie gevolgd door een intraperitoneale spoeling (H)IPEC met oxaliplatine. Deze patiënten werden verdeeld in 3 verschillende (H)IPEC groepen. Groep 1 zijn de patiënten die 90 min behandeld werden met 200 mg/m^2 oxaliplatine bij 37 °C, groep 2 bevat de patiënten die een 30 min durende behandeling kregen met 460 mg/m^2 oxaliplatine bij 37 °C en in groep 3 werden patiënten 30 min behandeld met 460 mg/m^2 oxaliplatine onder hypertherme omstandigheden (41 °C). Tijdens en na de (H)IPEC behandeling werden er perfusaat-, bloed- en weefselstalen genomen. In deze bloed- en

perfusaatstalen werd de hoeveelheid platinum bepaald door inductively coupled plasma-mass spectrometry (ICP-MS) en de platinum concentratie in de weefselstalen werd geanalyseerd door laser ablation ICP-MS (LA-ICP-MS). Postoperatief werd ook de mortaliteit en de morbiditeit geëvalueerd. Het vergelijken van de farmacokinetische parameters bij de normotherme en hypertherme behandelde patiënten (groep 2 vs. Groep 3) leverde geen significante verschillen op. Ook de platinum weefselpenetratie was voor beide groepen vergelijkbaar. Ondanks deze gelijklopende platinum concentraties vertoonden de klinische gegevens wel een verhoogde toxiciteit voor de patiënten behandeld bij 41 °C. In een tweede deel van de studie werd een korte behandeling met een hoge dosis oxaliplatine (groep 2) vergeleken met een lange behandeling met een lage dosis oxaliplatine (groep 1), bij beide groepen was de temperatuur identiek (37 °C). Bij de patiënten in groep 2 werd er een hogere maximale platinum concentratie waargenomen. Deze concentratie daalde echter zeer snel waardoor de $AUC_{t=7dagen}$ voor beide behandelingsstrategieën hetzelfde was. Daardoor werden er ook geen verschillen gevonden in de toxiciteit tussen beide groepen. De weefselpenetratie was significant hoger voor patiënten uit groep 2 in vergelijking met patiënten uit groep 1 (24.8 ± 13.2% vs. 2.7 ± 2.6%). Doordat de platinum weefsel concentratie veel hoger was voor de groep patiënten behandeld met een hoge dosis platinum terwijl dit niet resulteerde in een hogere toxiciteit, kan er geconcludeerd worden dat de dosis een groter effect zal hebben op de farmacokinetiek dan de perfusietijd.

De algemene conclusie aan het einde van dit onderzoek is dat hyperthermie geen toegevoegde waarde heeft voor de IPEC behandeling van patiënten met uitzaaiingen op het buikvlies. De dosis die gebruikt wordt tijdens de IPEC behandeling lijkt van cruciaal belang terwijl de perfusietijd eerder minder invloed lijkt te hebben. Alle geëvalueerde formulaties, Abraxane®, Genexol®PM en PTX/Plu F127, kunnen gebruikt worden voor IPEC behandelingen en zijn goede alternatieven voor Taxol®. Echter zal er in de toekomst nog verder onderzoek moeten gevoerd worden om IPEC behandelingen verder te optimaliseren. Ook dient er nagegaan te worden of er geen formulatie kan ontwikkelend worden specifiek voor intraperitoneale toepassingen. Een formulatie die een lange tijd in de buikholte kan verblijven terwijl ze continu hoge dosissen aan cytotoxisch geneesmiddel vrijstelt, lijkt het meest beloftevolle alternatief voor IPEC behandelingen.

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POSTER PRESENTATIONS

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Transformation of Itraconazole Nanosuspensions into Solid Dosage Forms, AAPS Annual Meeting and Exposition, Oktober 2012, Chicago

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In vitro evaluation of different paclitaxel formulations for the treatment of peritoneal carcinomatosis, 7th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, March 2010, Malta

ATTENDED WORKSHOPS, COURSES AND LECTURES

Personal effectiveness, Ghent University, 2011

Project Management, Ghent University, 2011

Introductory statistics, ICES, Ghent University, 2011

Analysis of variance, ICES, Ghent University, 2011

Advanced academic English: Conference skills, Ghent University, 2011

Advanced academic English: Writing skills, Ghent University, 2010

Design of experiments and multivariate data analysis basic course, Ghent University, 2008

Basic course in laboratory animal sciences, part 1&2, Ghent University, 2008